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CONTENTS

No. 1—January

	PAGE
JACKSON, S. H., T. F. NICHOLSON and W. L. HOLMAN, Carbohydrate metabolism and staphylococcus infection in rabbits	1
ODDY, J. G., and D. G. EVANS, The effects produced by toxic and non-toxic extracts of <i>H. pertussis</i> and <i>Br. bronchiseptica</i> on the blood sugar of rabbits	11
GORR, P. A., The incidence of tumours of the liver and other organs in a pure line of mice (Strong's CBA strain) (plates I and II) . .	17
GORR, P. A., Renal lesions found in pure lines of mice (plates III-VII)	25
STRAUB, M., The histology of catarrhal influenzal bronchitis and collapse of the lung in mice infected with influenza virus (plates VIII and IX)	31
OAKLEY, C. L., and G. HARRIET WARRACK, Immunity and antibody to influenza in mice	37
FISCHMAN, CATHERINE F., and DOROTHY S. RUSSELL, The occurrence of intranuclear inclusions in cultures of fatal leptomeninges (plate X)	53
NORMAN, R. M., and A. L. TAYLOR, Congenital diverticulum of the left ventricle of the heart in a case of epiloia (plates XI-XIV) . .	61
FLEMING, ALEXANDER, Observations on the bacteriostatic action of sulphamylamide and M & B 693 and on the influence thereon of bacteria and peptone (plate XV)	69
FAIRBROTHER, R. W., Coagulase production as a criterion for the classification of the staphylococci	83
WILSON, G. S., HERTA SCHWABACH and IRVING MAIR, The effect of the desensitisation of tuberculous guinea pigs	89
PAGEL, WALTER, The effect of blood serum on the growth of tubercle bacilli in the depth of liquid media	111
LI, PHILIP, Adaptation in veins to increased intravenous pressure, with special reference to the portal system and inferior vena cava (plates XVI-XVIII)	121
KROCH, E. V., and R. T. SIMMONS, Cultural methods as an aid in type differentiation of group A hemolytic streptococci	137
ISRAEL, M. C. G., The culture <i>in vitro</i> of leucocytes from human bone marrow (plates XIX and XX)	145
PIASECKA ZYLAND, E., and J. ZYLAND, Influence of the size of the infecting dose on the course of tuberculosis (plate XXI) . . .	153

RAVEN, CLARA, D. PEDEN and H. D. WRIGHT, The bacteriological examination of water supplies	287
KORENCHESKY, V., and K. HALL, Pathological changes in the sex organs after prolonged administration of sex hormones to female rats (plates XXXI-XXXV)	295
MULDER, J., <i>Haemophilus influenzae</i> and influenza virus in relation to bronchitis	317
COLLINS, DOUGLAS H., and WILLIAM GOLDIE, Observations on poly-arthritis and on experimental <i>Erysipelothrix</i> infection of swine (plates XXXVI-XLI)	323
WATTS, P. S., Studies on <i>Erysipelothrix rhusiopathiae</i>	355
BROWNING, C. H., PAUL BROWNING and J. V. M. Robb, A phenanthridinium compound of Morgan and Walls as a chemotherapeutic agent in experimental infections with <i>T. congolense</i> .	371
OAKLEY, C. L., and G. HARRIET WARRACK, The blood volume of the mouse	372
INNES, J. R. M., and J. W. WHITTICK, Thrombo-angiitis obliterans in a horse (plate XLII)	377
LENDRUM, ALAN C., and DAVID McFARLANE, A controllable modification of Mallory's trichromic staining method	381
BOOKS RECEIVED	385

No. 3—May

ORR, JOHN W., The histology of the rat's liver during the course of carcinogenesis by butter-yellow (<i>p</i> -dimethylaminoazobenzene) (plates XLIII-XLVII)	393
EDWARD, DERRICK G. FF., The occurrence in normal mice of pleuropneumonia-like organisms capable of producing pneumonia (plate XLVIII)	409
SHORT, R. H. D., The vasa vasorum of the femoral vein	419
BROWNING, PAUL, The chemotherapy of experimental staphylococcal infection in mice with drugs of the sulphanilamido group	431
FARRELL, L. N., and J. S. KITCHING, Passive protection of mice with immune horse serum against living staphylococci	439
INNES, J. R. M., DOROTHY S. RUSSELL and A. J. WILSON, Familial cerebellar hypoplasia and degeneration in Hereford calves (plates XLIX-LII)	455
PULLINGER, B. D., The first effects on mouse skin of some polycyclic hydrocarbons (plates LIII and LIV)	463
HAMILTON-PATERSON, J. L., and ELSIE W. M. JOHNSON, Post-mortem glycolysis	473
GORDON, J., and K. I. JOHNSTONE, The bactericidal action of normal sera	483

	PAGE
KHAIRAT, OMAR, The effect of a CO ₂ atmosphere on blood cultures	491
KHAIRAT, OMAR, Endocarditis due to a new species of <i>Hæmophilus</i> (plate LV)	497
COPLANS, M., Chemotherapy of the experimental typhoid and para- typhoid carrier condition in the immature rabbit	507
DUKES, CUTHBERT E., Cancer of the rectum : an analysis of 1000 cases (plates LVI-LX)	527
DACIE, J. V. The effect of iron deficiency on the fragility of rat erythrocytes	541
WITTS, L. J., Routine blood counts with heparinised blood	544
PERDRAU, J. R., The serological relationship between the viruses of Japanese and St Louis encephalitis	545
BONSER, G. M., and L. M. WAINMAN, Effect of a diet supplemented by fresh liver on induced skin tumours in inbred mice	548
BOOKS RECEIVED	551
INDEX OF SUBJECTS	553
INDEX OF AUTHORS	557

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CARBOHYDRATE METABOLISM AND STAPHY- LOCOCCUS INFECTION IN RABBITS

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It has been thought by many clinical observers that there is a relationship between staphylococcal skin infections and decreased sugar tolerance. The fact, known for many years, that diabetic patients are particularly subject to infection with staphylococci is the outstanding example of such a relationship. In such cases when infection does occur the intolerance of the individual to carbohydrate is greatly increased.

Since 1916 there have been numerous clinical investigations of the question. A number of investigators (Schwartz, Heimann and Mahnen, 1916; Seitz, 1919; Levin and Kahn, 1922; McGlasson, 1923; Haldin-Davis and Willis, 1925; Picard, 1927; Schmidt, Eastland and Burns, 1934) reported either raised blood sugar or a disturbance of glucose tolerance in various types of skin lesions. Other workers found no evidence of disturbed carbohydrate metabolism in similar types of cases (Loeb, 1926; Mannino, 1928; Nissle, 1929; Muller, 1929; Strickler and Saylor, 1929; Greenbaum, 1931; Rost, 1932; Tauber, 1933). In all these reports however various types of skin lesions were studied (acne, seborrhoea, sycosis, furunculosis) and in most of them no attempt was made to differentiate those due exclusively to staphylococci.

The experimental work on the relationship between carbohydrate metabolism and infection concerns itself with the transient hyperglycemia resulting from the injection of pathogenic bacteria or their products. This has been observed by a considerable number of investigators (Hirsch, 1921; Levine and Kolars, 1926-27; Evans and Zeckwer, 1927; Sweeney and his collaborators, 1928a, 1928b, 1934; Menten and King, 1930; Delafield, 1932).

* Part of the data embodied in this paper was taken from a thesis submitted by one of the writers (S. H. J.) in partial fulfilment of the requirements for the degree of Ph.D. in the University of Toronto.

In an endeavour to bridge some of the gaps between the extremely limited experimental findings and the clinical conditions encountered in practice and to find a more satisfactory explanation for the diversity of clinical findings, an investigation of experimental staphylococcal skin infections in rabbits was undertaken. It has already been mentioned that infections, as a complication in diabetes, show a marked tendency to lower the already impaired ability of the patient to handle ingested sugar.

In view of this observation and the controversy regarding the effect of infections on the carbohydrate of normal individuals it appeared possible that, when the ability of the organism to metabolise glucose was already impaired by some extraneous factor, the additional effect of the infection might produce a more readily observed result. For this reason the effect of staphylococcus infections was studied both in normal rabbits and in rabbits in which a certain degree of intolerance for glucose had been produced. Some unpublished work by Holman and Miller indicated that such an intolerance could be produced by prolonged feeding on a high carbohydrate diet.

Methods

Fully grown adult rabbits were used throughout the experiment. Considerable difficulty attended the selection of a diet sufficiently high in carbohydrate which the rabbits would eat without forcing for a sufficient period of time. The following feeding schedule was found to fill the requirements most satisfactorily. The amounts given are sufficient for 6 rabbits. On Mondays and Thursdays the ration consisted of $\frac{1}{4}$ lb. of cane sugar, $\frac{1}{4}$ lb. of oatmeal, $\frac{1}{4}$ lb. of ground Purina rabbit chow* and 1 yeast cake. This was kneaded with sufficient water to moisten it so that it formed balls but was not pasty. On Tuesdays and Fridays the rabbits were given about 5 medium-sized potatoes, chopped up and mixed with $\frac{1}{4}$ lb. of powdered cane sugar and $\frac{1}{4}$ lb. of ground Purina rabbit chow. On Wednesdays the diet supplied 5 medium-sized carrots chopped and mixed with $\frac{1}{4}$ lb. of powdered cane sugar and $\frac{1}{4}$ lb. of corn starch. On Saturdays a supply of oats was placed in the cages, sufficient to last over Sunday. At all times a supply of fresh 5 per cent. glucose solution was available for drinking. About 100 c.c. of this solution per rabbit per day were consumed. An important item in the diet was the adherence to a strict routine of feeding times. The rabbits were always fed first thing in the morning and any food remaining in the cage was removed at 5 p.m. If this was not done the animals lost their appetites. This diet supplied each rabbit with an average of 200 calories per day. Calculated on the dry weight it contained 89 per cent. carbohydrate, 10 per cent. protein and 1 per cent. fat, and supplied 0.29 g. of protein nitrogen per kg. per 24 hours. The rabbits, after the feeding

* A commercial product made up of chopped alfalfa, wheat-germ meal, soya bean oil meal, maize-germ meal, wheat middlings, crushed oats, cracked maize, crushed barley, blackstrap molasses, calcium carbonate and iodised salt. It contains protein 14.04 per cent.; fat 2.58 per cent.; fibre 13.44 per cent.; ash 6.04 per cent.; nitrogen-free extract 53.03 per cent.; Ca 0.741 per cent.; P 0.337 per cent.; K 0.967 per cent.; Na 0.367 per cent.; Mg 0.192 per cent.; S 0.268 per cent.; Cl 0.454 per cent.; vitamin A 1500 Sherman units per lb.; vitamin B 350 Sherman units per lb.; vitamin D a small amount; vitamin E abundant; vitamin B₁ 400 Sherman units per lb.

period of 8 weeks, seemed to be in good health and appeared entirely normal in every respect. Their average weight was the same as or slightly greater than at the beginning.

As a measure of the effect of the diet and the infections on the carbohydrate metabolism the glucose tolerance test was employed. A blood sample of 0.3 ml was taken from the ear vein after 12-16 hours' fasting. Three g of glucose per kg body weight were administered by stomach tube at zero time in the form of a 30 per cent solution. After $\frac{1}{2}$, 1, $1\frac{1}{2}$, $2\frac{1}{2}$ and 3 hrs, 0.3 ml blood samples were again taken. Sugar in these was determined with the Harding Downs (Harding and Downs, 1933) reagent on a Herbert and Bourne (1930) filtrate, the results representing true glucose.

RESULTS

That the high carbohydrate diet did produce a decrease of tolerance to glucose is clearly shown in fig 1, where a composite curve obtained from 14 rabbits on the "normal" diet is plotted with that obtained after eight weeks on the high carbohydrate diet.

Considerable difficulty was encountered in obtaining a consistent lesion by the subcutaneous injection of cultures of staphylococci. This was finally overcome by mixing, just prior to injection, a 24 hour culture of *Staph aureus* in Hartley's broth with an equal amount of melted 2 per cent agar which had been cooled to about 50° C. This mixture, while still liquid, was injected intradermally into the back of the animal, about 0.2 cc into each of 20 places. The agar was not absorbed and apparently acted as a reservoir for bacteria which finally broke down the resistance of the surrounding tissue.

This technique produced in normal rabbits spreading areas of necrosis of the skin, several of which often became confluent. They were indurated and bordered by a narrow margin of reddening in which dilatation of the capillaries could be observed. There was little or no production of free pus. The maximum effect was obtained in about 6 days, after which the dead skin sloughed off and healing commenced. The area was usually completely healed in about 3-4 weeks. Microscopically necrosis of the skin and subcutaneous tissues was observed, with a narrow surrounding band of leucocytic infiltration.

Such infections varied in their effect on glucose tolerance. Of the 14 cases recorded, 9 showed decreased tolerance (fig 2a), 2 were indefinite (fig 2b) and 3 showed increased tolerance (fig 2c). Of the 9 cases showing decreased tolerance, 5 remained within normal limits but 4 gave abnormal curves. It was possible to duplicate the curve obtained just prior to infection with considerable accuracy.

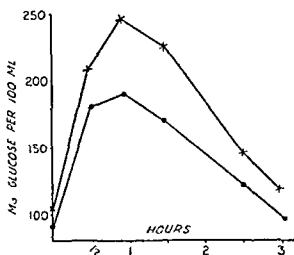


FIG 1—Composite glucose tolerance curves before and after a high carbohydrate diet.
 'Normal' diet ————— ●
 High carbohydrate diet - - - - - x

after the infection had cleared up, so that the variations observed during the infection could be interpreted fairly closely. The stage of the infection appeared to have little effect on glucose tolerance, the curve obtained after sloughing of the necrotic skin being sometimes higher, sometimes slightly lower and sometimes the same as that obtained before sloughing had occurred. The effect on the fasting blood sugar was very slight. A small increase in the average was observed but this was well within the biological variation and could be regarded as significant only because of the associated intolerance shown in the curves.

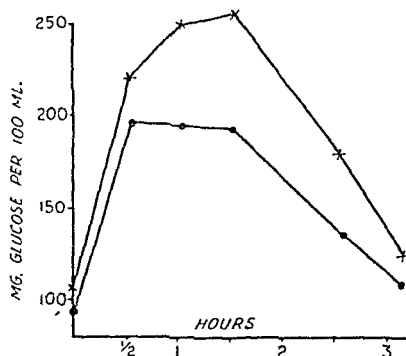


Fig. 2a

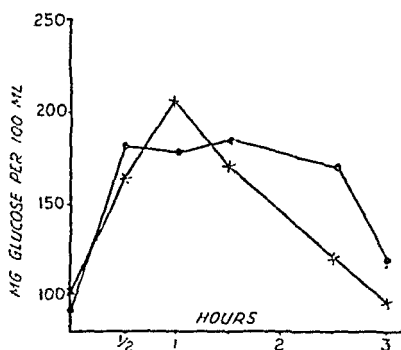


Fig. 2b

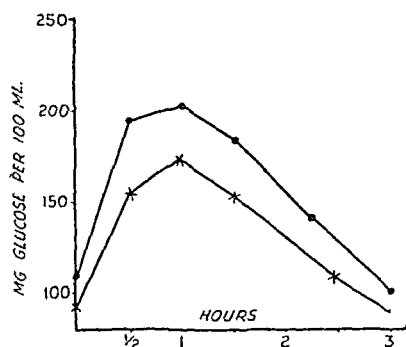


Fig. 2c

FIGS. 2a, 2b, 2c. — Composite glucose tolerance curves during intradermal staphylococcal infection in normal rabbits. 2a, decreased tolerance (9 rabbits); 2b, no change (2 rabbits); 2c, increased tolerance (3 rabbits)

Before infection —●—●—
During infection —×—×—

In rabbits which had received the high carbohydrate diet for 6-8 weeks a different type of lesion was obtained in the majority of cases (9 out of 13). This took the form of a large swollen lump filled with free pus. Necrosis was limited to a small head at the summit of the swelling, the remainder of the surface being reddened. In fact the lesion was similar in gross appearance to the common boil occurring in man. Microscopically there was a localised area of necrosis of the subcutaneous tissue filled with a dense mass of leucocytes and surrounded by an area of engorged capillaries. At the end of about a week the abscess broke down but the period

of discharge was short. The reddening disappeared and the skin over the lump became scaly but did not slough off as in the normal rabbits.

This type of lesion had a considerably different effect on the tolerance of the rabbits for glucose. In 8 out of the 9 cases (fig. 3a) the curve obtained prior to infection, already high owing to the diet, was reduced, often to well within normal limits. In the 4 cases (fig. 3b) which developed a type of lesion corresponding

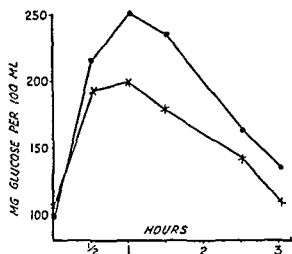


FIG. 3a

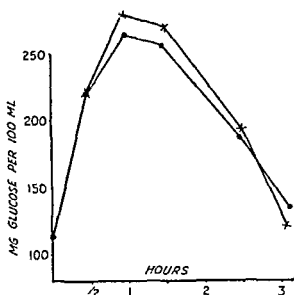


FIG. 3b

FIGS. 3a and 3b.—Composite glucose tolerance curves during staphylococcal infection in rabbits on a high carbohydrate diet. 3a, increased tolerance (9 rabbits), 3b, slightly decreased tolerance (4 rabbits)

Before infection ———●———
During infection ———×———

to that found in normal rabbits, the effect was if anything to raise the level of the already high curve still further. This was most marked in the case which showed most necrosis and spreading of the lesion.

Effect of staphylococcus toxin

The effect of intradermal injections of staphylococcus toxin was studied both in normal rabbits and in rabbits which had received the high carbohydrate diet. 0.1 c.c. of a 1:20 dilution of toxin was injected intradermally in each of about 20 places on the back. The resulting lesion was the same in gross appearance in both groups. There was typically a considerable area of necrosis surrounded by a narrow border of reddened tissue. There was no induration and the necrotic areas were soft and somewhat œdematous. This stage was reached in 3-4 days. Microscopically the lesion was the same in both groups of rabbits and showed necrosis of the superficial tissues with little or no leucocytic infiltration.

Figs. 4 and 5 record the results obtained in normal and dieted animals respectively. In normal rabbits there was a consistent slight loss in weight, although the animals appeared in good general condition. There was also a small but consistent decrease in glucose tolerance (fig. 4). In the rabbits on the high carbohydrate diet the injection of the toxin, while producing a similar skin lesion to that of the normals, had a marked effect on the general

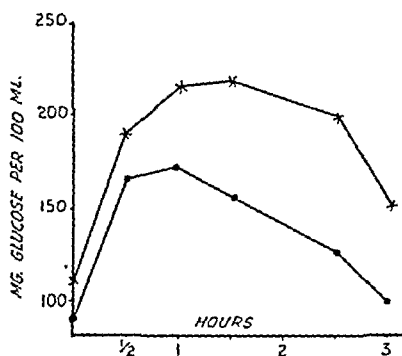


FIG. 4

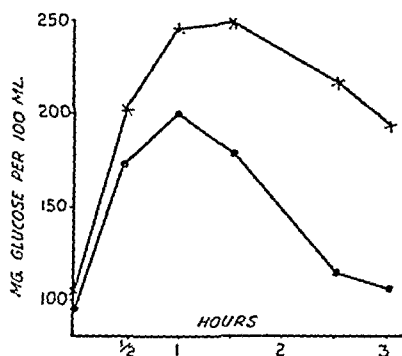


FIG. 5

FIG. 4.—Composite glucose tolerance curves following intradermal injection of staphylococcal toxin in 12 rabbits on a normal diet

FIG. 5.—Composite glucose tolerance curves following intradermal injection of staphylococcal toxin in 8 rabbits on a high carbohydrate diet

Before staphylococcal toxin —●—
After staphylococcal toxin —x—

well-being of the animals, which became listless and inactive and in some cases very weak. There was also a considerable loss of weight. Of the 8 cases included in fig. 5, 6 showed a decreased tolerance, while 2 were indefinite. The effect was generally more marked than in the normal rabbits.

Effect of necrosis by burning

Because of the possibility that the results in the animals on normal diet might be due to the absorption of toxic products from the necrotic tissue, an area of about 100 sq. cm. of skin on the backs of several rabbits was burned by the momentary application of a hot copper plate while the animals were under ether anaesthesia. Neither a light superficial nor a deeper burn had any effect on the tolerance of the rabbits for glucose 48 hours later.

Effect of turpentine injection

Since leucocytes have a high glycolytic activity and this might affect the glucose tolerance in the above experiments where pus was produced, an attempt was made to produce subcutaneous

abscesses by injecting old turpentine intradermally into the back, about 0.1 c.c. in each of 20 places. Unfortunately free pus was not produced. The typical lesion was similar macroscopically to that produced by staphylococcus toxin. There was a central area of necrosis of about 2 sq. cm. surrounded by a deep border of reddening in which individual distended capillaries were readily observable. This area was slightly oedematous but there was no production of free pus. Microscopic examination of sections showed necrosis of the superficial tissue with some leucocytic infiltration around the borders of the necrotic area. In this respect the lesion differed from that produced by the injection of staphylococcus toxin, but closely resembled that resulting from the intradermal injection of *Staph. aureus* in normal rabbits. The reaction to the turpentine reached a maximum in 3-4 days and was completely healed in 2 weeks.

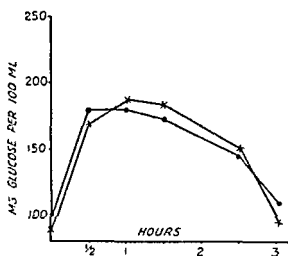


FIG. 6

FIG. 6.—Composite glucose tolerance curves following intradermal turpentine injection in 12 rabbits on a normal diet

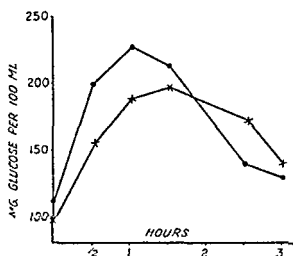


FIG. 7

FIG. 7.—Composite glucose tolerance curves following intradermal turpentine injection in 12 rabbits on a high carbohydrate diet

Before turpentine injection ●———●
After turpentine injection x———x

The turpentine had little effect on the general condition. Weight was consistently lost but the animals usually appeared well. In figs. 6 and 7 are shown the glucose tolerance curves of normal and specially dieted rabbits injected with turpentine. In neither group was any effect observed.

DISCUSSION

These results indicate that the effect of a staphylococcus lesion in rabbits on their carbohydrate metabolism bears a direct relation to the degree of localisation of the lesion. When the lesion is poorly localised so that a spreading necrotic area results, the

infection lowers the ability of the organism to metabolise glucose, whether in the normal rabbit or in rabbits which have been fed on a high carbohydrate diet. If, on the other hand, the lesion is well localised, with but a small area of skin necrosis and a local accumulation of free pus, the effect of the infection is, if anything, the reverse.

It seems possible from clinical reports that these observations on rabbits may be extended to man in a general way. Schwartz, Heimann and Mahnken found the most consistent disturbances in carbohydrate metabolism in cases of indurated acne. Schmidt, Eastland and Burns found the greatest abnormality in cases involving large, inflamed, pustular areas of infection. It appears also that the lack of consistency in the reported clinical findings can be explained in part by the divergent effects of these two general types of staphylococcus lesions.

The observation that a high carbohydrate diet will tend to better localisation of the infection agrees with Tauber's clinical finding. He gave intravenous glucose in a number of cases of furunculosis and reported remarkably good results.

These effects on carbohydrate metabolism appear to be related to the elaboration and absorption of toxin or other bacterial products from the infected areas. Toxin injections in both normal and high carbohydrate-dieted animals result in a necrotic area and a decrease in the tolerance for glucose. This is not an effect of the necrosis *per se*, since a similar necrosis induced by burns or by turpentine was without effect. Indeed the only observable difference, macroscopically or microscopically, between the staphylococcus lesion in normal rabbits and the turpentine lesion was the absence from the latter of bacteria and hence of bacterial products. Yet the former lesion produced a decreased glucose tolerance while the latter had no effect.

SUMMARY

Prolonged feeding with a high carbohydrate diet produces a degree of intolerance for glucose in rabbits.

It also results in a better localisation of experimentally induced staphylococcal skin infections, resulting in swelling with little superficial necrosis, whereas a spreading necrotic area with little swelling is produced by a similar technique in rabbits on a normal diet.

The localised infections increase the tolerance for glucose in rabbits, while the spreading type causes decreased tolerance. This decrease would appear to be due to the absorption of toxin rather than to the necrosis *per se*.

The thanks of one of us (S H J) are due to the Banting Research Foundation for a grant during part of the time spent on this work.

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612.352.12:615.372:576.851 (*Hæmophilus pertussis*):
576.851 (*Br. bronchiseptica*)

THE EFFECTS PRODUCED BY TOXIC AND NON-TOXIC EXTRACTS OF *H. PERTUSSIS* AND *BR. BRONCHISEPTICA* ON THE BLOOD SUGAR OF RABBITS

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IN an earlier paper Evans and Maitland (1937) described a method of preparing a toxic extract from *H. pertussis*. A suspension of bacilli was made in water, killed by alternate freezing and thawing and then dried *in vacuo* and the dried material ground in an agate mortar, resuspended in water and centrifuged at a high speed. The clear supernatant fluid was highly toxic, killing guinea-pigs in doses equivalent to 0.02 mg. dry weight per 100 g. body weight and producing necrosis in the skin of a rabbit or guinea-pig after the intradermal inoculation of doses as low as 0.3×10^{-3} mg. The toxicity was easily destroyed by heat. Extract heated at 55° C. for 30 minutes produced no necrosis after the intradermal injection of 0.3 mg. and large doses failed to kill guinea-pigs after intravenous injection. The extract also contained the bacterial antigen, which remained unaltered when exposed to a temperature which destroyed the toxin and could be precipitated by immune serum without reducing the toxicity of the supernatant fluid.

In a later paper Evans and Maitland (1939) showed that an extract prepared in the same way from *Br. bronchiseptica* contained a similar toxin as well as the bacterial antigen of this organism.

In our previous investigations we observed that rabbits and guinea-pigs which were injected intravenously with a lethal dose of the toxic extract of either *H. pertussis* or *Br. bronchiseptica* developed convulsive movements before death. This condition resembled that which is associated with hypoglycæmia and suggested that the effect of the toxins on the blood sugar concentration should be examined. The present paper records the result of a preliminary investigation in rabbits.

Experimental

Four preparations were tested—toxic and detoxified extracts of both *H. pertussis* and *Br. bronchiseptica*. The toxic extracts were prepared as described by Evans and Maitland (1937, 1939) from freshly isolated strains grown on Bordet-Gengou medium and were preserved by drying from the

frozen state *in vacuo* and storing in ampoules sealed *in vacuo*. For use the dry product was dissolved in water in the proportion of 2 mg. per c.c. The potency of the preparation was determined by the intradermal injection of 0.2 c.c. of a series of dilutions into the shaved back of a rabbit, the end-point being the highest dilution which produced a necrotic lesion. With the toxic extracts used in these experiments the end-point varied from 1:640 to 1:2560, which represented a dose in terms of dry weight of 0.62×10^{-3} to 0.15×10^{-3} mg. of extract.

The detoxified extracts were prepared by heating the toxic extracts for 30 minutes at 55° C.; when these were injected intradermally undiluted they produced no necrosis.

Each of the four preparations was tested by the intravenous injection of a number of rabbits weighing 2.2-5 kg. Preliminary tests showed that with toxic preparations of *H. pertussis* a dose of 0.35 mg. per kg. body weight killed the animals in about 15 hours, a period suitable for the experiments. From the intradermal test it was found that the extract of *Br. bronchiseptica* was less toxic than that of *H. pertussis* and accordingly the dose employed was about 0.6 mg. The dose of detoxified extract of *H. pertussis* was approximately the same as that of the toxic extract, whereas with *Br. bronchiseptica* the dose was 1.10 mg.

Blood sugar determinations, carried out by the Hagedorn and Jensen method, were made immediately before injection and at varying times thereafter on blood from the marginal ear vein of rabbits which had fasted for 24 hours.

Results

The blood sugar levels are recorded in the table and figs. 1 and 2 show graphically the changes which took place in four rabbits, one from each group. After the injection of either the *pertussis* or the *bronchiseptica* toxic extract a hyperglycæmia occurred, the maximum being reached within 2-4 hours. A few hours later the blood sugar had fallen to the normal level and thereafter there was a further fall until death, which occurred 9-24 hours after injection. The increase above the initial level resulting from the injection of *pertussis* toxic extract, was 55-74 mg. per 100 c.c., whereas with *bronchiseptica* the increase was 67-144 mg. The lowest figures recorded during the hypoglycæmic phase were for *pertussis* 42-78 mg. and for *bronchiseptica* 45-85 mg. below the initial readings.

When the detoxified (heated) extracts were injected a similar hyperglycæmia was recorded, the highest levels being of the same order as with the toxic extracts. With *pertussis* the increase above the initial readings was 60-116 mg. and with *bronchiseptica* 54-117 mg. This hyperglycæmia was followed by a return to the normal level and then in all cases except one there was a slight hypoglycæmia during the remainder of the experiment, up to 23 hours after injection with *bronchiseptica* and 22-26 hours with *pertussis*. With *pertussis* the fall below the initial reading was 8-31 mg.; with *bronchiseptica* it was 9 and 16 mg. in two rabbits and no fall was recorded in the third.

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Professor J. McINTOSH
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LONDON, W. 1.

January, 1940.

TABLE
Blood sugar changes in rabbits following the intravenous injection of extracts of H. pertussis and Br. bronchiseptica

Rat/Lit no.	Inoculum	Dose mg/kg	Before injection	Blood sugar in mg per 100 c.c.																	Remarks					
				Hours after injection																						
				1	2	4	5	6	7	8	9	10	11	12	13	14	15	16	17	19		22	23	26		
<i>H. pertussis</i>				136	138	198	173	100	97	124	10	87			90	76	49		74							D 10 hrs
10	Toxic	0.32	138																							" 17 "
11	extract	0.33	118																							" 16 "
12		0.33	122																							" 9 "
13		0.33	118																							" 9 "
14		0.42	120																							S
15	Heated	0.32	124																							.
16	extract	0.32	100																							" "
17		0.32	122																							" "
20		0.34	130																							" "
<i>Br. bronchiseptica</i>				127	238	231	105	126	127	122	109	92	114	115	90	52	81	80	70	99	91	112	106	109		
24	Toxic	0.65	137																							D 17 hrs
25	extract	0.65	126																							" 22 "
26		0.65	130																							" 24 "
27		0.60	139																							" 12 1/2 "
31	Heated	1.10	102																							S
32	extract	1.10	118																							" "
33		1.10	120																							" "

D = dead, S = survived

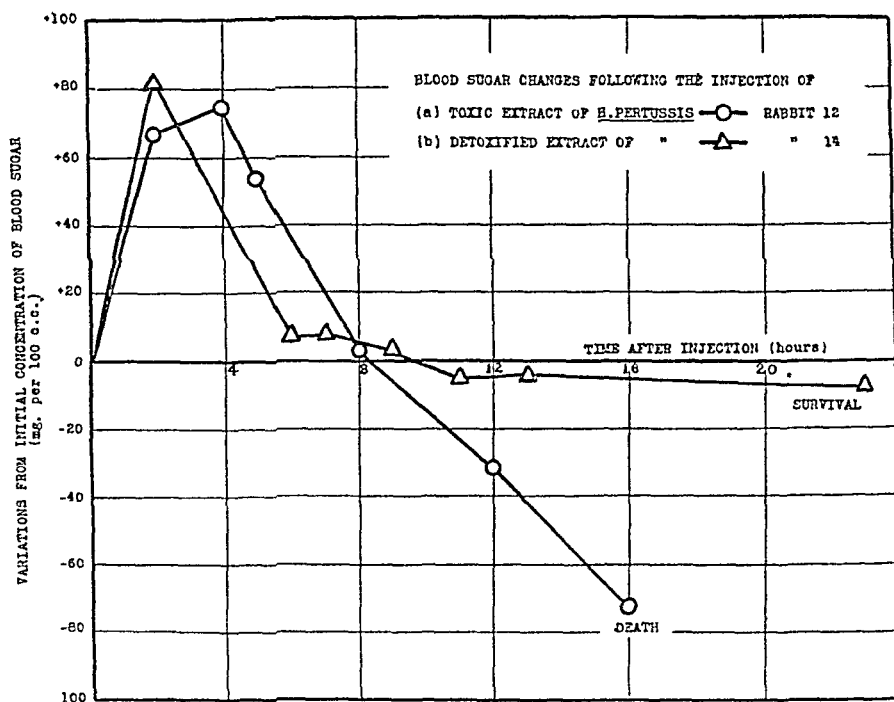


FIG. 1.—Blood sugar changes following the injection of (a) toxic and (b) detoxified extracts of *H. pertussis*

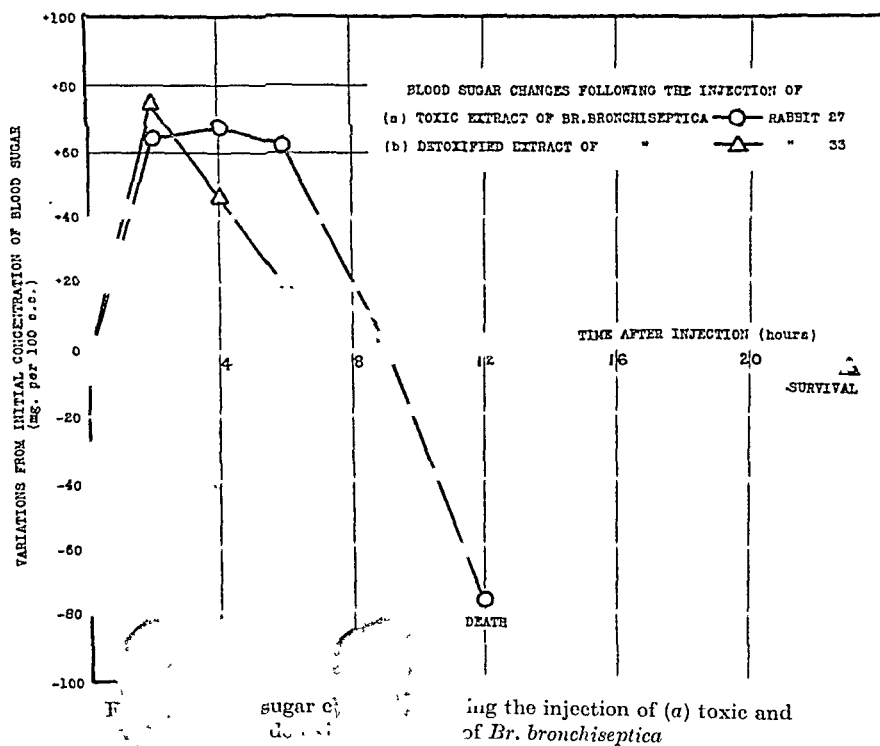


Fig. 2.—Blood sugar changes following the injection of (a) toxic and (b) detoxified extracts of *Br. bronchiseptica*

All the rabbits injected with the toxic extracts died in convulsions within 9-24 hours, whereas all those which received the detoxified extracts survived and had no convulsions.

Discussion

It is evident that a thermolabile as well as a thermostable factor was involved in producing the changes in the blood sugar concentration which followed the intravenous injection of the toxic extracts. The marked fall in blood sugar and death occurring in animals injected with unheated extracts were probably effects of the labile toxin. Heated non-toxic extracts produced a much smaller fall in blood sugar and the animals did not die. The initial hyperglycæmic phase occurred equally with heated and unheated extracts and it is not possible at present to specify a factor or factors to which this effect can be attributed. It seems evident that a thermostable substance is involved but there may be more than one and an initial hypoglycæmic action of toxin cannot at present be excluded. These extracts are known to contain a thermostable bacterial antigen and by analogy with the work of others it is probable that this might induce hyperglycemia.

DeLafield (1931, 1932), studying the changes in the blood sugar of rabbits following the intravenous injection of killed suspensions of various bacilli, found that 8 of the 12 Gram-negative organisms which he used produced hyperglycemia 2 hours after injection. In most cases there was a subsequent fall in blood sugar which at 24 hours was below the normal level. If the animals died they did so during the hypoglycæmic phase; if they survived, the blood sugar later returned to normal. The results which he obtained at 2 and 24 hours after injecting a killed suspension of *Br. bronchiseptica* agree in general with our results after injecting heated extract.

Bact. aertrycle was one of the organisms which DeLafield used in his earlier work (1931) and later (1934), using the stable purified antigen of this organism, which was toxic, he obtained the same type of response. The situation with regard to *H. pertussis* and *Br. bronchiseptica* appears to differ in some respects from that found for *Bact. aertrycle*. The marked hypoglycemia which occurred in our experiments was associated with thermolabile toxins and the hyperglycemia may have resulted from the heat-stable bacterial antigens which are relatively non-toxic.

We are not able to say that the death of rabbits and guinea pigs injected with toxic extracts was due directly to the hypoglycemia, although a low blood sugar level occurred regularly in rabbits at the time of death.

Summary

Intravenous injection of toxic extracts of *H. pertussis* and *Br. bronchiseptica* into rabbits causes hyperglycæmia followed by hypoglycæmia and death. With extracts rendered non-toxic by heating at 55° C. for 30 minutes, hyperglycæmia occurs but the subsequent hypoglycæmia is slight or absent.

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THE INCIDENCE OF TUMOURS OF THE LIVER AND OTHER ORGANS IN A PURE LINE OF MICE (STRONG'S CBA STRAIN)

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(PLATES I AND II)

THE origin of the CBA strain of mice is described by Strong (1936). The mice are wild type in colour and have been selected for longevity. Strong and Smith (1936a) noted the occurrence of liver tumours in this strain. Cloudman, Bittner and Little (1937) have given an account of the tumour incidence in breeding females kept at Bar Harbor, Maine, whilst Strong (1938 a and b) has given similar figures for mice kept by him at Yale. In these last communications special attention was paid to the effect of diet and general hygiene in increasing the expectation of life and thus the number of tumours found. When fed on a mixed oatmeal diet, tumours were found in 5.6 per cent of animals, the mean age at death for animals free from tumours being 502.4 days. A change of diet raised the tumour incidence to 12.7 per cent and the average length of life for non tumour mice to 639.5 days. In a third series of mice maintained on the new diet but kept with greater care than the mice of the first two groups, the tumour incidence was 37.0 per cent and the average length of life for tumour free mice 708.2 days. It seems clear that one effect on tumour incidence brought about by the change of diet may be attributed to an increase in the expectation of life, but, as Strong (1938 b, p. 89) points out, it is by no means certain that this is the only effect, especially as certain observations of Bittner (1935) indicate that dietary factors may influence the tumour incidence in the lower age groups. None of the above named authors appear to have published any data bearing on the effect of sex upon the tumour incidence in this strain. The following communication provides such data.

Materials and methods

Members of the CBA strain were brought from Dr Little's laboratory by Professor Haldane in 1932, at which time the animals had been brother-sister inbred for 27 generations, this type of inbreeding has been continued in this laboratory since their arrival. The mice were housed in wooden cages till the early part of this year, when a change to metal cages was made. The basis of the diet is Purina fox chow which is supplemented by a seed mixture and the diet is thus somewhat similar to that used by Little.

It is generally the custom in genetical laboratories to allow all animals to die a natural death or at most to kill them when they have a palpable tumour. This practice has many obvious advantages and indeed is essential for many purposes. However there are many situations in which it is

* In receipt of a whole time grant from the British Empire Cancer Campaign.

desirable to obtain an estimate of the number of animals likely to have tumours in any given age group. In the case of superficial growths such as mammary tumours one can observe how long the appearance of a tumour may precede death, but with internal tumours this is impossible. It will be seen that valuable information may be obtained from post-mortem findings upon animals that have died or been killed for various purposes. In this series of animals, a certain number were found dead, others were killed with palpable tumours, whilst some died during cardiac puncture or were killed to obtain organs for serological experiments, etc. Naturally animals bearing grafted tumours are not included in the report.

Results

In stating the tumour incidence in any group of animals, it is obvious that attention must be paid to the age at death. Some authors give the age of the animals to the nearest day. For most purposes this renders the data extremely puzzling and some system of grouping is clearly necessary. For very large groups of data a division into monthly or bi-monthly age groups is suitable, but for the data to be presented here tri-monthly groups have been chosen. This may appear to be a somewhat coarse system of classification but if smaller age groups are employed the number belonging to any one group is too small to give any useful information.

In table I are shown the findings for animals over the age of 11 months, no tumours having been observed in over 40 post-mortems upon animals dying at ages less than this. The most striking feature of the table is the great excess of tumours shown

TABLE I

The incidence of tumours in different age groups

Age in months	Males		Females		Total
	Non-tumour	With tumour	Non-tumour	With tumour	
11-13	6	0	4	1	11
14-16	8	6	8	2	24
17-19	2	4	5	2	13
20-22	1	4	11	3	19
23 and over .	0	4	5	2	11
Totals . . .	17	18	33	10	78

by males (approximately 50 per cent. as against 25 per cent. for females); indeed over the age of 17 months the tumour incidence in males tends towards 100 per cent. It should also be noticed that the age of death for females is definitely later than it is for males, which must tend if anything to raise the significance of the difference between the sexes. Of the 43 females, 14 were

breeders and the rest virgins. Four of the breeders developed tumours. It is not possible with these figures to say whether breeding affects the tumour incidence.

In table II the tumours are listed according to type. It will be noticed that 20 tumours occurred amongst 18 tumour bearing

TABLE II

Tumours of the CBA strain classified according to type

	Males	Females	Total
Liver tumours	14	3	17
Spindle celled sarcomata	1	2	3
Pulmonary tumours	4*	0	4
Angiomata	1	0	1
Leukoses †	0	4	4
Ovarian tumour		1	1
Totals	20	10	30

* One pulmonary tumour co existed with the angiomata another with a liver tumour

† Includes 3 leukaemias and 1 lymphosarcoma

males. In one case a pulmonary tumour occurred with an angioma, in another it co existed with a liver tumour. Histological examination excluded the possibility of the pulmonary growths being metastatic.

The most striking feature of the table is the incidence of liver tumours in the males. These growths which are formed from liver cells and not from bile ducts, have been described previously without reference to the incidence in the sexes by Strong and Smith (1936a) but further points may be raised here. The youngest animals to be found with hepatomas by Strong and Smith were 18 months old. In this series 4 out of 14 males examined between 14 and 16 months of age had liver tumours. The growths at this age were usually small and presumably would not have been fatal until some months later. In most cases the tumour is considerably lighter in colour than the surrounding liver and it is not surprising to find that numbers of the tumour cells are extremely fatty. Apart from these fatty cells, which are frequently small, very large vacuolated and non vacuolated cells are generally found, the tumours in general being markedly pleomorphic (figs 1-3). Multiple tumours are not infrequent and it is possible that some of these may be local metastases, but frequently the nodules are of roughly the same size. The tumours may cause death in at least three ways: they may grow to such a size that the rest of the liver is obliterated, they are extremely vascular and a fatal hæmorrhage may take place, and finally in one case extensive

pulmonary metastasis had occurred (fig. 2). Strong and Smith (1936*b*) have transplanted one of these tumours subcutaneously and another has been carried through 2 transfers within its own pure line in this laboratory (fig. 3). Both tumours grew very slowly and the growth employed in this laboratory took about 6 months to kill. It is also of interest to be able to confirm the finding of Strong and Smith (1936*a*) that these tumours are not usually associated with inflammatory changes in the rest of the liver.

There have been three cases of spindle-celled sarcoma, one in a male and two in females. That in the male (aged 21 months) arose in the spleen and had metastasised extensively to the liver. It was transplanted and grew very slowly but formed numerous metastases. Of those in females, one arose in the mesenteric chain of lymph glands of a breeding female, the other occurred in the uterus of a virgin.

The tumours classified as leukoses were confined to females. Three were definitely leukæmias, the fourth was a *lymphosarcoma* without blood changes. It is worth noting that leukæmia has been found in females only, in any of the pure lines kept in this laboratory. If the data for all lines are pooled this fact appears to be of statistical significance. However, it appears possible to induce leukæmia in males of some lines by methods to be described in a future paper. It is doubtful if the sex ratio for other growths is of any significance.

The earliest tumour to occur was an ovarian growth in a virgin aged 11 months and as it is a very unusual type of growth it is worth describing in some detail. It was originally contained within a cyst which was accidentally burst during removal. The growth consisted of a gelatinous papillary mass. Histologically it consisted of undifferentiated cells lying in a hyaline gelatinous matrix (fig. 4). Some parts of the tumour appeared to be almost free from cells. The tumour was successfully transplanted within the CBA strain but grew very slowly, the animals sometimes surviving a year following inoculation. Unfortunately the tumour was lost in a somewhat unusual manner. When the subcutaneous implant was examined, it was thought to be infected, so it was decided to use some peritoneal deposits; unfortunately these were found to be sarcomatous: the mouse, in fact, had a primary sarcoma of the spleen, which incidentally was not markedly enlarged. The only other ovarian tumour to be transplanted is that described by Strong and co-workers (Strong and Hill, 1937; Strong, Gardner and Hill, 1937; Strong, Pfeiffer and Gardner, 1938). This tumour was an *oestrogenic granulosa-celled carcinoma* which grew fairly readily in males but only exceptionally in females. The tumour described above agrees in no particular with that described by Strong. It showed no evidence of *oestrogenic* activity and grew

TUMOURS IN MICE

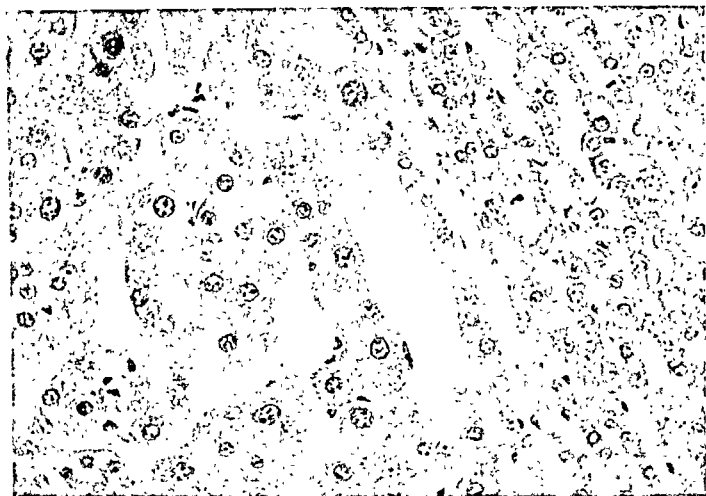


FIG. 1.—Portion of a liver tumour which formed numerous pulmonary metastases, showing an area of small vacuolated cells and one of very large vacuolated and non-vacuolated cells. The primary growth showed no histological features to distinguish it from non-metastasising growths. $\times 370$



FIG. 2.—Pulmonary metastasis from tumour shown in fig. 1. $\times 110$



equally well in members of either sex. It certainly was not a granulosa-celled tumour and it is very difficult to classify. Unfortunately its loss prevented further investigation.

Discussion

The most striking feature of these data lies in the sex ratio for liver tumours, the absolute number of other tumours being too small to justify any conclusions being drawn. For all ages over 11 months (the earliest age at which any tumour occurred) 40 per cent. of males developed liver tumours, but the latter type of tumour did not appear before 14-16 months and for all ages over 14 months the percentage incidence of liver tumours in males and females was 48 and 7.8 respectively. It is interesting to note that although all published details concerning the incidence of tumours in the CBA strain have dealt with females only, Strong, Smith and Gardner (1937-38) used males of this strain to study the effect of 3:4:5:6-dibenzcarbazole on the incidence of liver tumours. These workers found that liver tumours occurred in treated males 8 months old, which is certainly younger than any recorded here, but one would like to have control groups of untreated males in this type of experiment and to know the relative effects of the treatment on males and females.

Of the two other strains kept in this laboratory, Strong's A strain and Little's C.57 black strain, only the latter has given rise to liver tumours. Seven such tumours have been obtained up to date, 4 in males and 3 in females (the gross incidence is of the order of 5 per cent.). There is thus no indication that sex is of any importance in this strain so far as liver tumours are concerned. It is also of interest to note that Slye *et al.* (1915-16), Slye (1916 *a* and *b*) and Wells (1916) could find no evidence of any influence attributable to sex in the genetically heterogeneous groups of mice studied by them. The data thus appear very confusing for tumours of this type and it may be as well to consider the sex ratio of other mouse tumours, excluding mammary cancer or those affecting other sex organs.

Pybus and Miller (1938) find that sarcomata (mostly of the bones) are about 2.5 times more frequent in females than in males. Gardner and Pfeiffer (1937-38) found several striking effects exerted upon the skeleton by oestrogenic hormones (see also Allen, 1937-38) and the differential sex incidence might be explained along these lines. Unfortunately Wells (1916) and Slye *et al.* (1917) could find no sex difference in the incidence of sarcomata. Indeed 12 bone tumours were equally divided between the sexes.

A similarly puzzling picture may be found when considering the incidence of leukaemia, lympho-sarcoma and allied conditions.

In normal untreated animals from the three pure lines kept in this laboratory there have been 8 cases of leukæmia, all in females. Indeed there has been but one case of lymphosarcoma in a male although well over 100 have lived to the cancer age. About 140 females have lived to a similar age and if leukæmias and lymphosarcomata are considered together the preponderance of females is further emphasised. These findings are consistent with those of Mercier (1938) who found that 64.9 per cent. of 254 females and 34.1 per cent. of 211 males of a certain strain of mice developed lymphomatoses of one type or another. Furthermore there have been reports of conditions of this type following prolonged treatment with oestrogenic hormones (Lacassagne, 1938; Lawrence and Gardner, 1938; Lits *et al.*, 1938; Cramer and Horning, 1939). Unfortunately we are once more confronted with completely contradictory data from the intensively studied strain C.58 used by MacDowell (1936) and his co-workers. In this case 90 per cent. of the animals develop leukæmia and there is no indication that either sex is more prone to the disease.

It is quite possible that the entirely contradictory mass of data might be explained in terms of two variables, the bisexual potentialities of the sex hormones, and genetic variables which might affect the amount of hormones secreted but more probably the sensitivity of the various tissues to the hormones concerned. Until very recently practically all geneticists would have accepted the statement that the inter-strain differences in the incidence of mammary cancer were to be explained in terms of a genetically determined differential susceptibility to the action of the pertinent hormones. However, more recent work by Bittner (1939) in Little's laboratory has shown that this explanation is insufficient so far as mammary cancer is concerned; a third variable is of great importance, namely an agent passed in the milk. Two groups of mice genetically identical and treated in exactly the same way may or may not get cancer depending for the most part upon whether they have received the milk-borne agent. It is possible that similar agents, transmitted in a different way, may play an equally decisive part in the incidence of other types of neoplasia. If this is so geneticists will have to consider the question of susceptibility to such agents as well as to the important chemical stimuli recognised at present (*cf.* Gorer, 1937).

Summary

1. This paper deals with members of the CBA pure line of Strong that have come to post-mortem for a variety of reasons.

2. Of 35 males living for 11 months or over, 18 developed tumours; of 43 females surviving this time, 10 developed tumours. The average age at death was greater for females.

TUMOURS IN MICE

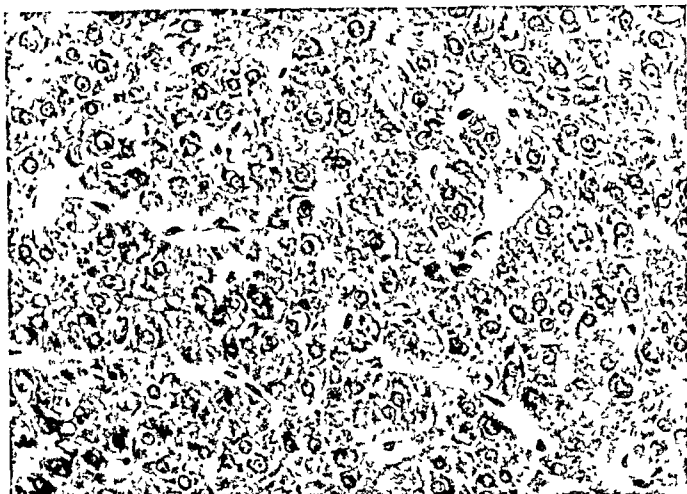


FIG. 3—Another type of cell frequently seen in liver tumours. The section is from a second generation transplant. The original growth had similar cells. $\times 370$

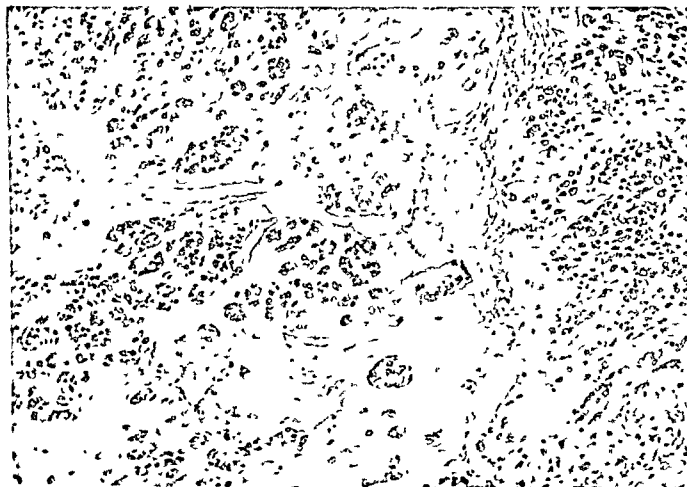


FIG. 4—General view of ovarian tumour. The matrix shows much hyaline change. $\times 110$

3 Fourteen males and three females developed liver tumours, one of which metastasised

4 In one male a lung tumour co existed with a liver tumour, in another with an angioma. There were thus 20 tumours in 18 tumour-bearing males

5 A liver tumour, a spindle celled sarcoma and an ovarian tumour have been transplanted. The last would grow in both males and females and showed no evidence of oestrogenic activity

6 The significance of sex differences in the incidence of mouse tumours is discussed in connection with genetically determined differences in susceptibility

It is a pleasure to acknowledge the encouragement and valuable advice given by Sir John Ledingham and Professor J B S Haldane

ADDENDUM

Since this paper was submitted for publication Andervont (1939) and Burns and Schenken (1939) have published data concerning the incidence of liver tumours in the C₃H strain of mice. In this strain liver tumours are also found more frequently in males, however the picture is complicated by the very high incidence of mammary carcinomata at a comparatively early age in both breeding and virgin females. It is worth noting that the C₃H strain is related to the CBA strain

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RENAL LESIONS FOUND IN PURE LINES OF MICE

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(PLATES III VII)

SINCE many people working with mice must have noticed the relative frequency of renal lesions in these animals it is surprising that nobody appears to have attempted to describe them Jaffe (1931) in his text book on the diseases of small laboratory animals gives no details of renal diseases in mice Maud Slye in her numerous communications on the incidence of neoplasms in mice frequently makes references to nephritis without describing the lesions further Andervont (1938) mentions the common occurrence of cystic kidneys in Strong's A strain and points out that such lesions were not observed in first generation hybrids between this strain and Little's C 57 blacks

Strong's A strain (albinos), Little's C 57 blacks and Strong's CBA (agouti) strains have been maintained in this laboratory principally for experiments with spontaneous and transplanted tumours Details of feeding and housing have been given in the preceding paper (Gorer, 1940) The animals have come to post-mortem for a number of different reasons Many of them had spontaneous neoplasms, but those bearing transplanted growths are not included in the records Since the main purpose of the records has been concerned with the cancer problem it did not appear justifiable to attempt a highly detailed study of the lesions found

Renal lesions found

Three main types of lesion were found in the pure lines, the incidence of two of which differs in these three lines It must be emphasised, however, that all three lesions may co exist in the same kidney

(a) *Glomerular metaplasia* One of the most striking features of the mouse kidney is the frequency with which the parietal layer

* In receipt of a whole time grant from the British Empire Cancer Campaign

of the glomerular capsule undergoes metaplasia to cubical or even columnar epithelium. The condition has been found in young mice suffering from infections of various kinds. It has also been found in an apparently healthy pregnant female that was near full term, the foetuses being alive and in excellent condition. Only a few kidneys from pregnant females have been examined and all except one were normal. Although this particular lesion is not confined to any one strain, it was observed most frequently in old members of the CBA strain. Figs. 1 and 2 are taken from the kidneys of an old male from this pure line. It will be noticed that many of the glomerular tufts appear to be lying within tubules. In some cases the capsule may somewhat resemble that seen in human nephritis with the characteristic crescents, but this appearance is not particularly common and may merge into the metaplasia described above.

(b) *Cystic disease.* Cystic kidneys are by no means rare in mice derived from a variety of sources but, as has been pointed out by Andervont (*loc. cit.*), are specially common in the A strain. The earliest stage to be seen macroscopically is the presence of cysts or depressions upon the surface of the kidney. Another characteristic and early change is a peculiar brown colouration; this feature is, however, not invariably present. A typical cystic kidney is shown in fig. 3.

Histological investigation makes it fairly certain that the disease is a special type of hydronephrosis and it may be that the human congenital polycystic kidney is of similar origin. In early cases it can be seen that there is focal necrosis of the tips of the renal papillæ (figs. 5-8). In later stages these necrotic areas are usually not seen; probably they slough off as appears to be happening in fig. 8. The effect on the rest of the nephron is somewhat variable, depending perhaps upon the degree of obstruction produced. In typical cases the tubules dilate first (fig. 4), this dilatation being most marked in the convoluted tubules; dilatation of the glomerular capsule occurs later. In other cases the formation of cysts is not a prominent feature, atrophic changes dominating the picture from the beginning. It is not uncommon to find that one kidney closely resembles a congenital cystic kidney whilst the other is reduced to a tiny fibrous remnant. The rest of the kidney in early stages may show patches of round-celled infiltration and metaplasia in some of the glomerular capsules.

The origin of the necrosis in the renal papillæ is at present obscure. It is perhaps worth mentioning in this connection that members of the A strain are liable to internal hæmorrhages, usually into the lumen of the gut. These may be fatal but recovery has been observed, and indeed one female had three litters subsequent to such hæmorrhage.

RENAL LESIONS IN PURPURIC MICE

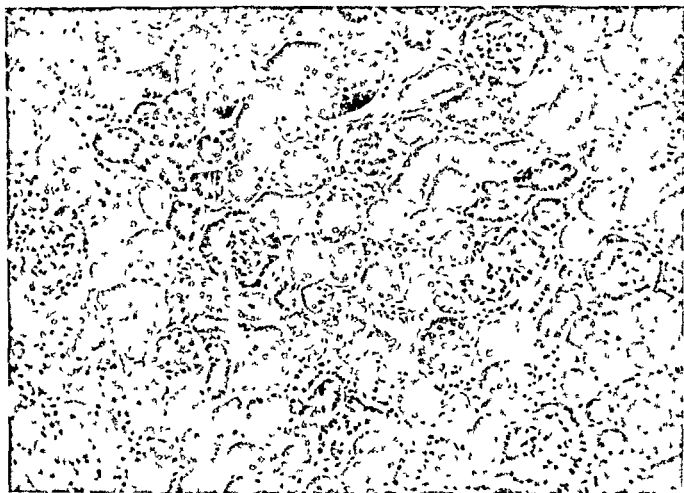


FIG. 1—Low power view of kidney showing extensive metaplasia of the glomerular capsules $\times 160$

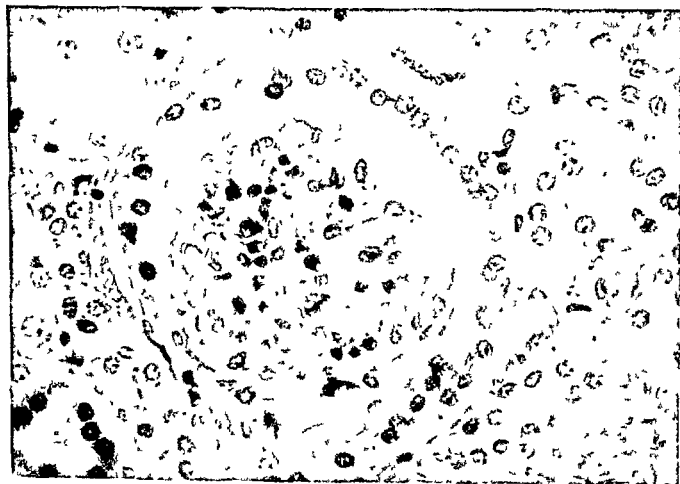


FIG. 2—High power view of a single glomerulus showing columnar epithelium lining Bowman's capsule $\times 770$

RENAL LESIONS IN PURE LINE MICE

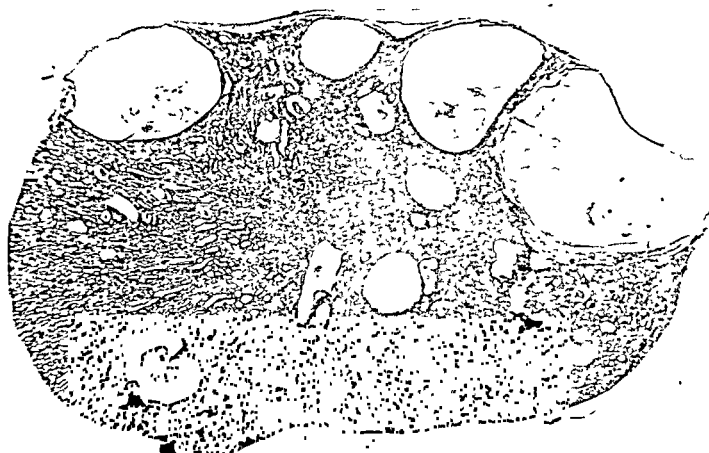


FIG. 3.—General view of a cystic kidney of an A strain mouse. Note widespread dilatation of tubules. $\times 12$

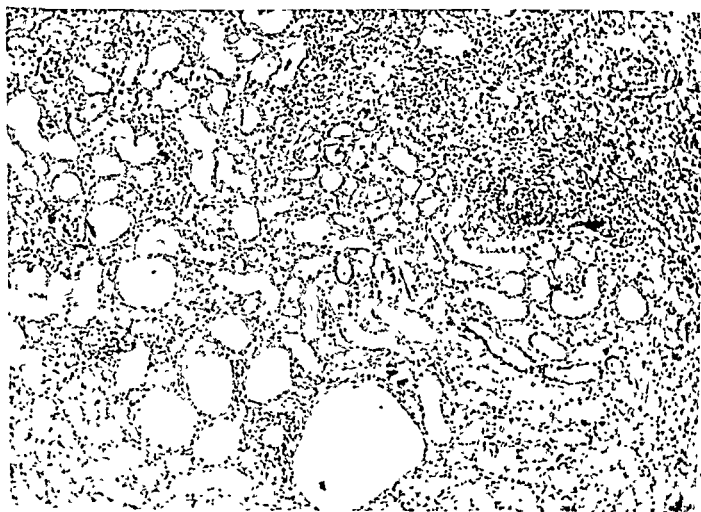


FIG. 4.—Dilatation of renal tubules. The glomerular spaces are not yet dilated. $\times 110$

The accompanying table shows the incidence of this disease in the A strain as diagnosed macroscopically. This table does not include all post-mortems for this strain, since in many cases no diagnosis of the condition of the kidneys was possible owing to decomposition. It will be seen that above the age of 16 months nearly 100 per cent. of members of this strain have visibly diseased kidneys. It is therefore not surprising that this strain is relatively

TABLE

The incidence of macroscopic cystic renal disease in A strain mice

	Age of mice in months					
	11-12	13-14	15-16	17-18	19 and over	Totals
Affected . . .	4	8	13	12	11	48
Normal . . .	10	4	4	1	0	19
Total . . .	14	12	17	13	11	67

short-lived. The best figures for the mortality within this strain are given by Bittner (1939) and the data obtained in this laboratory indicate that similar conditions exist here. Mammary cancer is a potent cause of death in breeding females, but in virgins this cause of death is of negligible importance and in males it is absent. Actually Bittner found that virgin females have a higher expectation of life than males. Ninety per cent. of males were dead at 20 months, whilst 90 per cent. of virgin females were dead at 24 months.

Typical cystic disease has been found in members of the C. 57 black strain but never in the CBA strain, although 43 members of the latter have lived 17 months or over. There have been 135 post-mortem examinations of blacks living 11 months or more. Cystic disease was diagnosed macroscopically in 10 mice, and two other cases were found on histological examination. The youngest mouse with cystic disease was 19 months old, whilst 8 out of the 12 cases observed were over two years old. There is no need to apply any statistical tests of significance to these figures.

One case of cystic kidney occurring in an old black mouse is not included in the above figures. In it the glomerular tufts were enormously enlarged and the cysts originated in the glomerular capsules and not in the tubules.

(c) *Hyaline degeneration*. This condition is illustrated in figs. 9 and 10. Up to the present it has only been observed in members of the black pure line. The youngest animal in which it has been observed was 18 months old. It may occur earlier

but since it can only be diagnosed histologically, more data are required for the younger age groups. It is doubtful whether really extensive lesions are common much under the age of two years. In 9 kidneys examined histologically from animals aged 17-22 months extensive lesions were observed once and early lesions twice. Of nine kidneys from animals aged 23-25 months 3 were normal and 6 diseased, whilst 4 animals aged 26 months or over all showed the condition. Thus amongst 22 animals aged more than 17 months 59 per cent. showed this lesion. In some cases it coexisted with cystic disease. Fifteen kidneys of members of the CBA strain aged 17 months or over have been examined histologically and none showed the condition, nor has it been observed in the A strain.

This lesion shows considerable resemblance to amyloid disease but fails to give intense staining with iodine or metachromatic staining with methyl violet or gentian violet. It begins in the glomerular tufts, but in later stages it may be seen as an exudate between the tubules. The liver and spleen have not yet been observed to show a comparable lesion, but changes in the suprarenals sometimes appear very like it. Such changes appear to start in the medulla and to spread peripherally.

None of the three lesions mentioned above appear to occur more frequently in either sex.

Discussion

The data presented above offer many points of genetic interest. Andervont points out that cystic disease is not observed in F_1 hybrids between the A strain and C. 57 blacks kept in his laboratory. If the disease were influenced by the presence of a milk-borne agent one would expect it to occur in hybrids derived from albino females. In some respects its relative incidence in the different strains is very similar to that shown by tumours of various kinds. As has been pointed out elsewhere (Strong, 1936; Gorer, 1937), pure lines do not fall sharply into resistant and susceptible classes so far as spontaneous tumours are concerned, and a comparison of the incidence of cystic renal disease in the albinos, blacks and agoutis indicates an analogous state of affairs. This demonstrates that a single gene cannot be solely responsible for the high incidence shown by the albinos, but of course it may be that a single gene accounts for a great deal of the difference between albinos and blacks. Thus it may be that the latter pure line is homozygous for a gene giving about a 10 per cent. incidence over the age of 18 months; the presence of a second gene might raise the incidence to the albino level. It may be that the agoutis lack both these hypothetical genes. Owing to the ease with which this disease can usually be diagnosed its genetic basis could probably be

RENAL LESIONS IN PURL LINE MICE

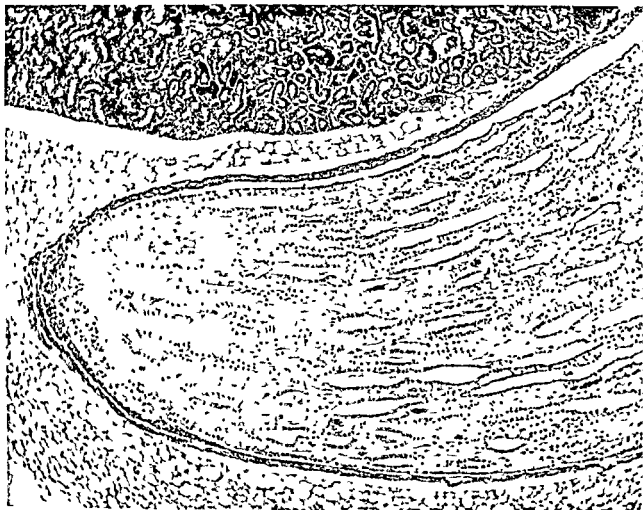
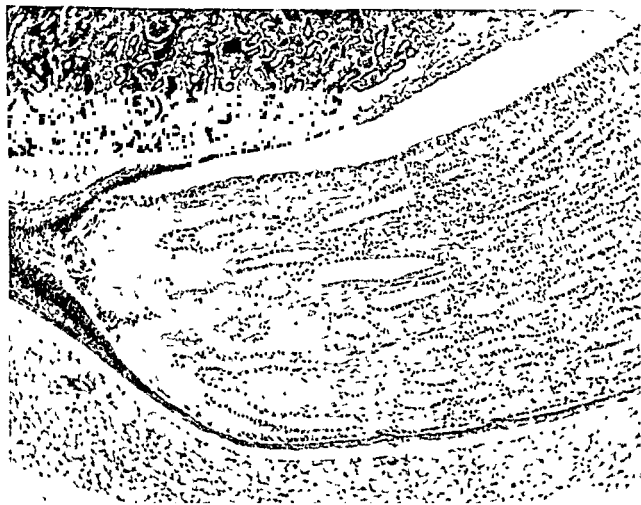


FIG. 5—Very early stage of necrosis of tip of renal papilla $\times 90$



RENAL LESIONS IN PURE LINE MICE



FIG. 7.—Completely necrotic tip of renal papilla, which however still stains intensely with eosin. $\times 90$



FIG. 8.—Commencing demarcation of necrotic area, which now stains $\times 6$.

determined with some accuracy. The late age of incidence of the hyaline change, together with the fact that histological diagnosis is essential, makes the genetical analysis much harder.

From the point of view of cancer research these diseases of the kidney are by no means without importance. Obviously liability to renal disease at an early age may prejudice the tumour incidence, but there is a further point where a short expectation of life may influence opinions on the genetics of cancer. With an internal tumour such as a lung tumour it is difficult or impossible to decide how long the appearance of the tumour precedes death. In the case of the A strain, lung tumours are commonly found *post mortem*, but as a rule—at any rate in this laboratory—the growths cannot be implicated as a cause of death, which is usually associated with renal disease. A small number of F₁ hybrids between albinos and blacks have come to post-mortem here and cystic renal disease has not been found; the animals frequently live over 2 years. Amongst the older animals examined lung tumours are commonly found, and there can be no doubt that their average size is considerably greater than those found in albinos at an earlier age. At present, therefore, we cannot say whether lung tumours arise about the same time in hybrids and in the pure line. Factors such as these may easily cause confusion in discussing the question of the dominance of susceptibility to internal tumours.

The incidence of renal disease may prove of importance in connection with chemotherapy. Strong (1939) found that methyl salicylate had a pronounced inhibitory effect upon spontaneous mammary carcinomata arising in members of the CBA strain but not upon those of the A strain. Mice of the former strain were more tolerant of the drug than those of the latter, which may well be explained by the presence of renal disease in A strain mice.

Summary

Three types of renal disease have been found in members of three pure lines of mice (Strong's A strain, Little's C. 57 blacks and Strong's CBA strain) coming to post-mortem for a variety of reasons.

1. In mice of any strain the parietal layer of the glomerular capsule is liable to undergo metaplasia to a cubical or columnar form. This lesion has been found most often in old members of the CBA strain.
2. Members of the A strain are liable to develop cystic or atrophic kidneys; the disease may appear at 11 months, and by 18 months almost every member of the strain has visibly diseased kidneys. A few members of the blacks

strain show similar lesions, but the youngest case was at 19 months; it has not yet been observed in the CBA strain. This disease appears to be a special type of hydronephrosis; obstruction of the nephrons appears to be brought about by focal necrosis at the tips of the renal papillæ.

3. Members of the black strain aged more than 18 months frequently show lesions of the glomeruli (and occasionally of the tubules) resembling amyloid but failing to give the typical staining reactions for this substance.

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RENAL LESIONS IN PURE LINE MICE

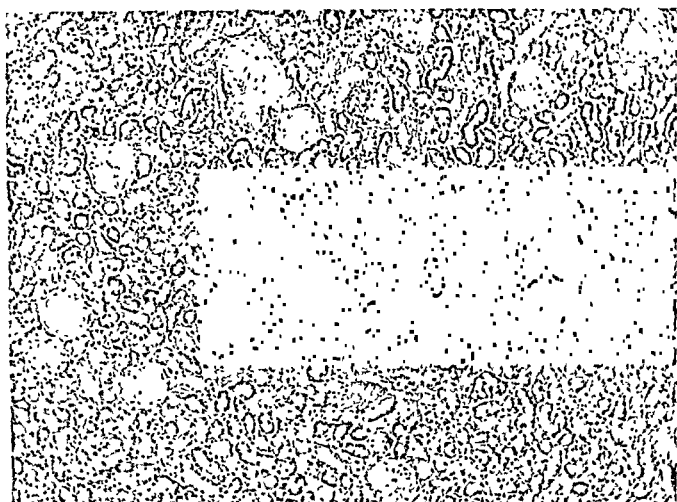


FIG. 9.—Hyaline degeneration of glomeruli. $\times 110$

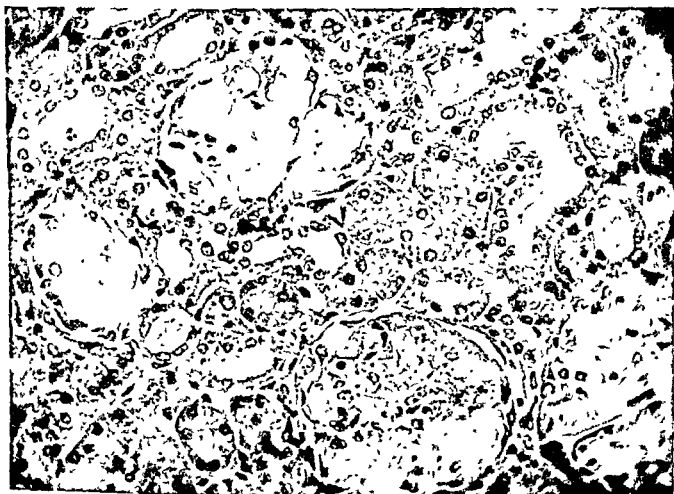
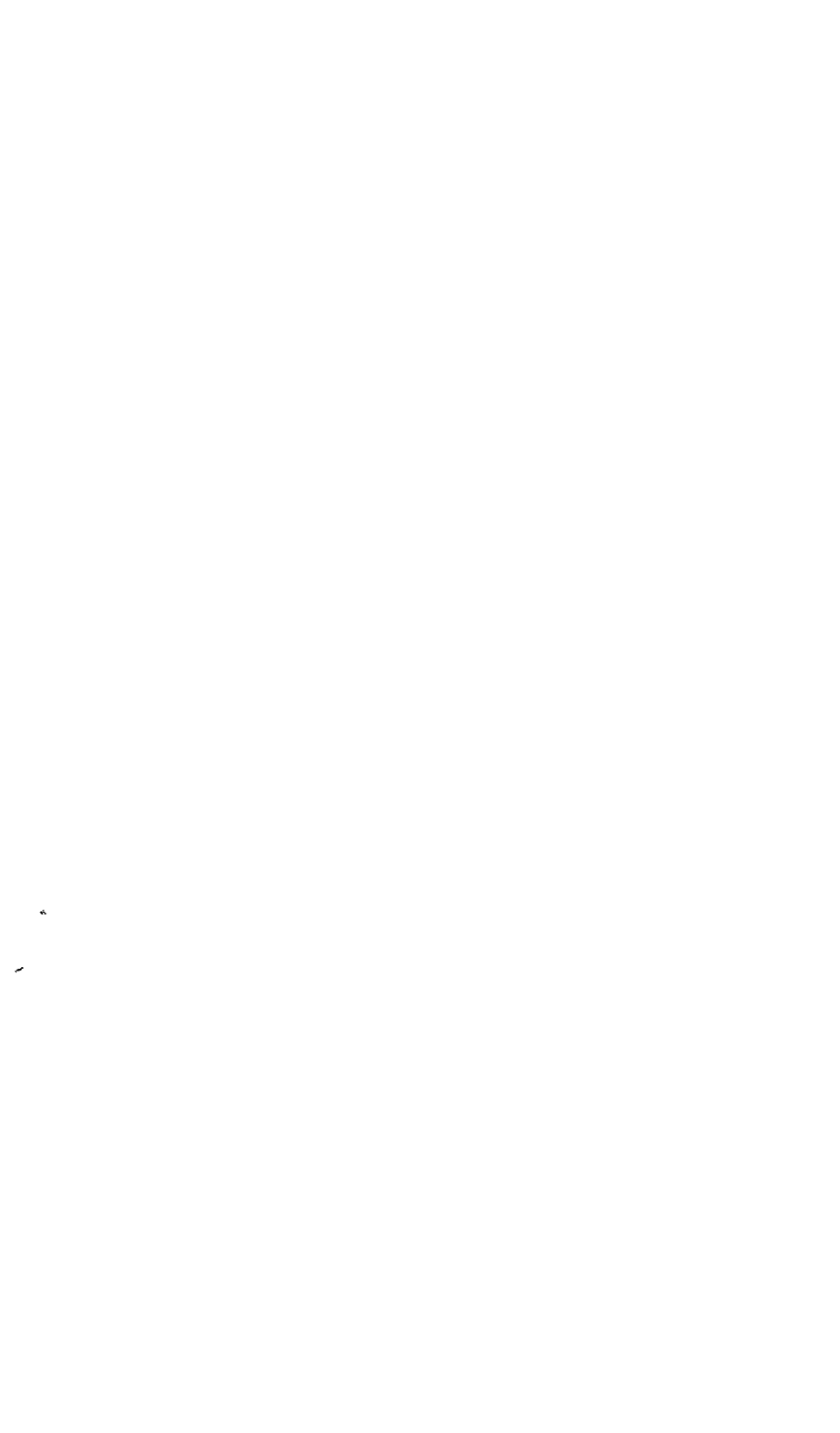


FIG. 10.—Higher power view of kidney of an old member of the C. 57 black strain showing varying degrees of hyalinisation of glomerular tufts



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THE HISTOLOGY OF CATARRHAL INFLUENZAL BRONCHITIS AND COLLAPSE OF THE LUNG IN MICE INFECTED WITH INFLUENZA VIRUS

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(PLATES VIII AND IX)

In a paper contributed to this *Journal* (Straub, 1937), I described changes found in the lungs of mice after infection with human influenza virus, laying particular stress on the changes in the epithelial covering of the bronchioles. Necrosis and proliferation of this epithelium seemed to me more or less specific for influenza in mice. I left it an open question whether, in these animals, *restitutio ad integrum* of early epithelial lesions was possible.

Further observations allow a more complete description of the whole epithelial process following infection with human as well as swine virus.* In this paper special attention is given to the condition of the larger bronchi, the study of which seems to be of importance for a better understanding of the action of influenza virus in general. The histological results of infection with weak virus and the effect produced by virus infection of adult mice are also discussed. In conclusion a few remarks concerning immunity to influenza virus in mice are given.

In order not to disturb anatomical relations in the larger bronchi, infected mice, after partial dissection, were fixed whole in formalin, the thorax being opened only after fixation.

Although on the *first* day after infection with the filtrate used the mice do not betray a single symptom of disease, the bronchial epithelium from the bronchioles to the bifurcation of the trachea displays evident reaction. Normally consisting of one row of low columnar cells covered by a thin layer of mucus, it now appears to be considerably broadened. The halves of the cells closest to the bronchial lumen are swollen from the presence of mucoid droplets (fig. 1; swine virus). When the more virulent human virus is used this stage is not always so clearly visible, because transitions to the later stages manifest themselves early. Both

* Both viruses were kindly put at my disposal by Dr C. H. Andrewes.

viruses, however, produce similar changes in the nuclei of the cells, which are flattened and stain darkly with the nuclear dye. They have become pyknotic. Scattered between these pyknotic nuclei other nuclei remain unaltered.

After *two* days the dark nuclei have moved towards the lumen of the bronchus, the unaffected nuclei retaining their place at the base of the cells. A layer of mucus with disintegration products of nuclei is observable on the damaged epithelium. These products are for the most part remnants of epithelial cell nuclei, to some extent also of polymorphonuclear leucocytes.

On the *third* day after infection the epithelium shows evidence of still more serious damage. An indefinable layer of cells, alive and necrobiotic, is found (fig. 2; human virus). After *four* days all that is left of the lining of the larger bronchi is one layer of flattened epithelium (fig. 3). This seems to be formed by those cells which, even on the first day after infection, were distinguished by their unaltered nuclear structure. The others have entirely vanished. Their remains are to be found in the thick layer of mucus now covering the bronchial wall.

In *four* days, with the virus used, the degenerative process in the large bronchi is accomplished and regeneration starts.

Two or *three* days after infection, areas of collapse begin to form in the lungs. Accumulations of mucus occasion total occlusion of bronchi and in its train collapse of lung tissue. This occurs first in the areas around the hilus, whose bronchi are short and narrow. Elsewhere, in the better ventilated parts of the lungs, emphysema may occur. There is no doubt that the crepitating sound during respiration, the first symptom of disease in infected mice, is caused by the abnormal contents of the larger bronchi. Later the collapse spreads from the hilus to entire lobes. Of pneumonia there is no question anywhere. The condition of the bronchi is to be apprehended as a purely catarrhal inflammation. The changes in the lung tissue seen naked eye are only secondary in nature and must not be mistaken for a specific process.

On the *fifth* day the mice are very ill owing to the extent of the collapsed areas. They become more and more cyanotic. In the end, death ensues with convulsions, as in suffocation. Yet, in spite of the steadily developing morbid symptoms, the bronchial epithelium begins to reveal signs of powerful regeneration. On the fifth day after infection a layer of several rows of cells with many mitoses covers the bronchial wall (fig. 4). Regeneration evidently originates from the flattened epithelium found on the fourth day. When the mouse dies on the *sixth* or *seventh* day after infection a complete epithelial layer has been formed (figs. 5 and 6). It still consists of several rows of cells; it often resembles stratified squamous epithelium and displays a number of mitoses.

VIRUS INFLUENZAL BRONCHITIS



FIG. 1.—Z. 552. Bronchial epithelium one day after infection with swine virus. $\times 455$.

FIG. 2.—S. 450. Bronchial epithelium three days after infection with human influenza virus. $\times 460$.



FIG. 3.—S. 451. Bronchial epithelium four days after infection with human influenza virus. $\times 460$.

FIG. 4.—S. 454. Bronchial epithelium five days after infection with human influenza virus. $\times 460$.



VIRUS INFLUENZAL BRONCHITIS



FIG. 5.—Z. 20. Bronchial epithelium six days after infection with swine virus. $\times 312$.



FIG. 6.—S. 448. Bronchial epithelium seven days after infection with human influenza virus. $\times 460$.



FIG. 7.—S. 1140. Bronchial epithelium one day after infection with weak human influenza virus. Two mitotic figures. $\times 453$.

Experiment 1. Of eighteen control mice, one week after infection with weak virus, only three displayed slight typical lung lesions. Of eighteen other mice, treated with diluted diphtheria toxin one or two days before infection with the same virus, fifteen showed extensive lung lesions.

Diphtheria toxin in the dilution used, even when applied twice with an interval of one or two days, causes no appreciable macroscopical lesions, and microscopically one finds only a slight catarrhal lesion of the bronchial epithelium. The summation of both agents, diphtheria toxin and virus, each in itself of slight importance, proves to be able to produce similar diffuse changes, by means of stagnation of bronchial secretion, to those caused by strong virus alone. These observations make it clear how virus can be transferred from one mouse to another in the absence of distinct macroscopical lesions. Collapse of lung tissue is merely a secondary phenomenon.

In order to obtain constant results the use of young mice in experiments with influenza virus is recommended. By means of a little artifice it is possible to prove that adult mice are as liable to be infected as young ones. This is done by instillation intranasally under ether narcosis of an indifferent fluid one or two days after infection with the virus. I used Tyrode solution.

Experiment 2. A virus filtrate, very active in young mice, effected no clinical signs of disease in six four-months-old mice weighing $22\frac{1}{2}$ g. Ten days after infection two showed no lung lesions at all and two only slight lesions; in the remaining two the lesions were rather extensive.

Of six mice, in all respects resembling these others, treated with Tyrode solution two days after infection with the same virus, only one remained unaffected, the five others dying within eight days with very extensive and typical lesions.

Experiment 3. Two groups each of six four-months-old mice, of an average weight of $21\frac{1}{2}$ g., were infected with a strongly active filtrate of human virus. The second group was treated with Tyrode solution two days later. After five days the mice of the first group were in good health, with an average weight of 19 g. Post-mortem examination showed, in two only, uncertain changes near the hilus of some lobes. A filtrate of these lungs proved to be inactive for young mice. In the second group the weight at the end of five days had been reduced to an average of 15 g. and the mice were very ill. The lungs displayed unmistakable and in four cases very extensive lesions. A filtrate of these lungs was active for young mice.

In mice treated with virus and Tyrode the epithelial lesions are similar in character to but more severe than those in the controls. Collapse of lung tissue consequently occurs, but in addition intra-alveolar pneumonic changes are found. If the animal survives, a few months later the lungs show only slight areas of epithelialised chronic collapse. The greater part of the changes found with X-rays fourteen days after infection have totally cleared up. This is contrary to what we see in young mice treated with virus only

and is in my opinion an argument in favour of not looking upon this pneumonia as a specific virus pneumonia. The products of the catarrh in the larger bronchi are washed with the Tyrode to the periphery where they cause non specific and as a rule sterile pneumonia, with a number of polymorphonuclear leucocytes. Occasionally there arises a state of things resembling the "Swiss cheese lung," not unknown in cases of human malignant influenza.

However this may be, the after treatment with Tyrode is a means of aggravating the epithelial lesions and of rendering demonstrable the extensive bronchial catarrh caused by the virus. As with young mice the secondary collapse indicates activity of the epithelial virus, so here the secondary pneumonic changes have a like significance. Tentatively a comparison can be made with human influenza and its bacterial complications.

The question now suggests itself whether, with one of the artifices mentioned above, it is possible to overcome influenza immunity in mice. This proved not to be the case.

Experiment 4 Of 12 mice infected with virus filtrate and given after treatment with Tyrode, 10 died within six days the two survivors displaying extensive typical lesions. With the same virus 24 adult mice were infected, 14 survived. Twelve of the latter were reinfected and given Tyrode after treatment two months later. Within six days three were dead and one, on the seventh day, showing extensive typical lesions. In eight no fresh lesions were found even microscopically. It was only these eight which displayed epithelialised areas, sometimes very slight, attributable to the first infection, the first four were free from older lesions.

This experiment favours the view that immunity is here due to the presence of the older lesions. In that case the chronic epithelial changes found in the lungs of mice after infection with influenza virus appear to us in a different light. They are not exclusively a phenomenon of organisation or an example of disturbed recovery; they have a function to perform. From a teleological point of view they might be apprehended as a useful phenomenon. Only a serious catarrhal infection leaving metaplastic epithelial changes is able to produce immunity. The harmless catarrh recovering without leaving a trace, gives no immunity. This observation may lead to a better understanding of catarrhal immunity.

Summary

1 In mice, influenza virus affects specifically the epithelium of the respiratory tract from the bronchioles to the bifurcation of the trachea. It causes catarrhal bronchitis with collapse of lung tissue. This collapse is merely a complication of the epithelial process and not in itself specific. It may be absent after infection

with weak virus or in adult mice in which case treatment with diluted diphtheria toxin prior to infection or treatment with Tyrode solution one or two days after infection is capable of aggravating the consequences of the diffuse catarrhal inflammation present.

2. In influenzal bronchitis, after a degenerative stage there follows a regenerative stage of the epithelial process. In the larger bronchi, this entails *restitutio ad integrum*: in the bronchioles, metaplastic epithelial changes may occur.

3. Immunity to influenza virus in mice depends largely upon the presence of such epithelialised areas. Even very slight metaplastic change seems to give complete protection.

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IMMUNITY AND ANTIBODY TO INFLUENZA IN MICE

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BURNET, Keogh and Lush, in their monograph on the immunological reactions of the filterable viruses (1937), put forward the possibility that antibody to viruses might be produced locally in the structures for which the virus has a special affinity and suggested that influenza, with its localisation, as far as was then known, in lung and turbinate, was a very suitable virus on which to work to determine this point. We therefore immunised mice in various ways in the hope of demonstrating antibody in the lungs before it appeared in the circulation.

We immunised them (1) by inoculation intranasally under anaesthetic with a dose of virus just sufficient to infect * but not to kill, (2) by vaccination with two intraperitoneal doses of living virus at an interval of about one week and (3) by vaccination in a similar way with formalised virus. We killed them at times which we thought appropriate, bled them out from the heart, washed out the vessels with warm citrate saline by Welcker's method and determined the antibody in serum and organs against the homologous strain of virus, using throughout as standard the serum of a ferret convalescent from infection with this strain. We have used in all this group of experiments the WS strain, as it is remarkably constant in its behaviour in mice.

Antibody in serum

Though it is well known that antibody to influenza virus appears in the serum after infection in the ferret (Smith, Andrewes and Laidlaw, 1933), man (Francis, 1934-35, Andrewes, Laidlaw and Smith, 1935, Francis and Magill, 1935, Francis, Magill, Beck and Rickard, 1937), hedgehog (Stuart Harris, 1936), rat and guinea pig (Stuart Harris, 1937), Chinese mink—*Mustela sibirica*—(Tang, 1938), little work has been done on the time of its appearance or its persistence. Fourteen days after infection large amounts are present in the serum of ferrets (Smith and Andrewes, 1938) and traces can be detected as late as 12 months, though much of the immunity has faded (Stuart Harris *et al.*, 1938). Stuart Harris *et al.* also found that antibody after natural infection in man appeared in the serum in about 8 days, reached its maximum in 9-12 days and could still be detected 52 days after infection. Rosenbusch and Shope (1939) have shown that antibody to swine influenza virus appears in pigs about 7 days after infection, reaches a maximum on the 14th-27th day and persists in some pigs though at a rather diminished titre for at least 84 days.

* As will be seen later (p. 38) the meaning of this word is not as clear as might appear.

A great difficulty in our work has been to determine a dilution of W.S. virus which would infect but not kill. Smith, Andrewes and Laidlaw (1935) have previously noted this difficulty. It is not unusual for a 10^{-5} dilution of filtered virus to kill every mouse inoculated, some dying as late as 3 weeks with completely consolidated lungs, while a 10^{-6} dilution may produce no lesions in any mice. It is only with great good luck that a dilution is hit on which will infect 75 per cent. of the mice and kill only 5-10 per cent. We have been forced to give our results for intranasal inoculation in a rather incomplete form, up to 3 months only,

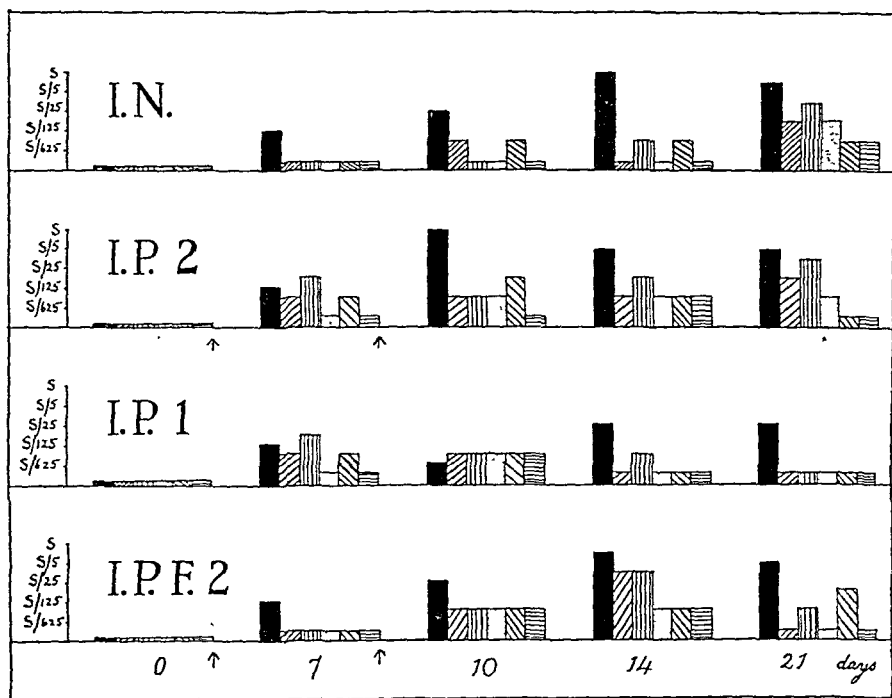


FIG. 1.—Development of antibody in mice immunised in various ways to W.S. influenza virus

I.N. = by intranasal inoculation of 10-100 M.I.D. of virus

I.P.2. = with two intraperitoneal doses of 0.2 and 0.5 c.c. of living virus (10^7 M.I.D. per c.c.) at an interval of a week

I.P.1. = with one intraperitoneal dose of 0.5 c.c. of living virus

I.P.F.2. = with two intraperitoneal doses of 0.2 and 0.5 c.c. of formolised virus (10^7 M.I.D. per c.c.) at an interval of a week

In each group the values given are (from left to right) for serum, nose, lung, liver, spleen, kidney.

rather than use up the immense number of mice required for a complete series. Recently at Dr Andrewes's suggestion we have used the neurotropic variant of the W.S. strain with a good deal of success, but we feel that it is not quite the same as mouse-lung-

adapted virus, and that comparison of mice inoculated intranasally with neurotropic influenza virus with mice vaccinated intraperitoneally with lung virus is hardly fair. We have found in a few experiments that antibody to lung virus after infection with neurotropic virus is lower than that after infection with lung virus, suggesting that differentiation is already occurring.

In man, Francis and Magill (1937) have shown that after vaccination with living culture virus, antibody appears in the 2nd week and persists about 5 months. Stuart-Harris *et al.* report the development of antibody in mice after vaccination with living

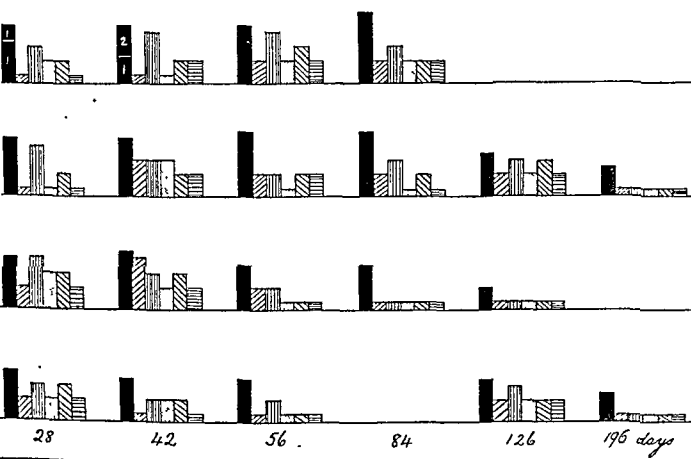


FIG. 1—continued

virus. Our results are given in fig. 1, which shows the values for antibody in serum, nose, lung, liver, spleen and kidney of groups of 5 or more mice at various times after immunisation.

Owing to the logarithmic method of expressing the results and the great variation in the response to infection in mice, the answers obtained from pooled samples of serum tend to be weighted in favour of high values. For instance if one serum has a titre equal to standard (S) and four a titre of S/125, the result for the pooled sera will be S/5; ordinarily it is not practicable either to use a large enough number of mice to avoid this difficulty, or to carry out serum titrations on individual mice.

As might be expected from the effect of a secondary stimulus on antibody titre in immunity against other antigens, the rise in

antibody is fastest in animals receiving two doses of living virus intraperitoneally, and the greater part of the rise occurs soon after the second dose. It is therefore surprising that the rise in animals receiving two doses of formolised virus is no faster than in those infected intranasally. All reach the same maximum in about 14 days,* none so far showing much advantage.

The disappearance of antibody is very different in the three groups. We have previously held the view that antibody due to intranasal inoculation is reduced to negligible proportions in about six weeks but further work has shown that this is true only of some groups of mice: at 28 days after infection two groups showed serum antibody equal to S/25 and S/5; in three others the titres at 42 days were S/25, S/5 and S/5. In many sera the concentration is high up to three months, beyond which time we have not been able to carry the experiment.†

By about 8 weeks antibody due to vaccination with formolised virus has largely disappeared, while that due to vaccination with living virus is still at a high level at 12 weeks—a considerable time in the life of a mouse.

As we thought that the high values for some groups at and after 6 weeks might be due to deterioration of the standard (Smith and Andrewes), we tested all the sera from the groups infected intranasally at one time against the same standard. We obtained much the same result as before.

The values given in fig. 1 for antibody after infection apply only to those animals showing lesions in the lung. Mice showing no lesions after "infection" almost invariably have no demonstrable antibody.

As a matter of interest we determined the antibody response in mice vaccinated with one dose of living virus intraperitoneally; the antibody levels were always low, rising only on one occasion to S/5 and thereafter remaining at about S/25.

Antibody in organs

Before examining the results some consideration of their accuracy is necessary. The method adopted was to wash out first the lungs and then the systemic circulation until the washings were colourless, grind up the organs with quartz powder and ten times their weight of broth, freeze overnight, thaw and allow the gross particles to settle, and having determined the antibody titre in the supernatant in the usual way against 100 M.I.D. of W.S. virus, multiply the answer by ten. It not infrequently happened that even undiluted organ extracts failed to protect; all that could be said of such extracts was that their titre was $<S/3125$ or $<S/15625$ according to the efficiency of the standard; the corrected value for the organ

* Compare the results of Stuart-Harris *et al.*, p. 116, for man.

† Compare the irregular peaks in antibody observed by Stuart-Harris *et al.* in ferrets.

had to be set down as $<S/300$ or $<S/1500$, though its real titre might easily have been $S/20,000$. Moreover it is possible that Welcker's method may not wash all the serum out of the vessels: if 1 per cent. is left behind and the serum value is S , the organs, even if they contained no antibody, might give a corrected titre of $S/100$.

We feel that in organs only titres greater than $S/100$ should be allowed any weight; and almost the only organs with this or a higher titre are the lungs, with occasionally the nose or spleen. The spleen is notoriously difficult to wash out, so that, though the result is interesting, we do not lay much stress on it. The only other organs with a significant if small amount of antibody are the lungs and nose, the ordinary sites of election of the virus. It will be observed, however, that when the lungs are rich in antibody so is the serum; we have thus no evidence that antibody is produced in the lung. If it is, it must be very rapidly removed from the lungs to the circulation.

We see no way of getting over the difficulty that, as the animal is washed out, antibody normally more or less fixed in the tissues may be removed: our methods of estimating antibody are far too inaccurate to permit of any determination of this point.

Antibody and the persistence of virus in tissues

In animals infected intranasally, W.S. virus persists in diminishing concentration in lung and turbinate for 7-14 days. At this time antibody reaches its highest concentration in the serum; later no virus can be demonstrated and no further rise in antibody occurs.

After intraperitoneal vaccination with sufficiently large amounts of living virus, virus can be demonstrated in the lungs; typical influenzal lesions are sometimes produced and occasionally death (Rickard and Francis, 1938). Virus persists for 7-11 days after the first inoculation and for a shorter time after the second. No increase in antibody occurs after the disappearance of virus.

After intraperitoneal inoculation with formolised virus no living virus can be detected, but antibody reaches its maximum at about the same time as in infection or vaccination with living virus. The apparent connection between time of maximum antibody level and time of disappearance of virus may therefore be coincidence.

Antibody and immunity

Infection or vaccination of the susceptible animal is followed by the development of antibody and immunity, but this immunity varies much in quality as well as in degree. Ferrets convalescent from intranasal infection with a given strain are apparently immune for a considerable time to all other strains, though they may have

significant amounts of antibody only for the homologous strain. Mice vaccinated with living or formolised virus show greatest immunity to the homologous and closely related strains and very little to serologically unrelated strains (Smith and Andrewes). The picture is much complicated by questions of the titre of virus used for vaccination and test inoculation (Francis, 1939).

We have therefore investigated the relationship between immunity and antibody by immunising groups of mice with W.S. virus either by infecting them intranasally with small doses of living virus or by vaccinating them intraperitoneally with two doses of formolised virus, to avoid the possible effect of the localisation of living virus in the lung. At appropriate times thereafter some of them were bled for antibody, the blood from those showing lesions in the lungs being kept separate from that of those showing none. Suitable numbers of the rest were inoculated intranasally with falling tenfold dilutions of the four main strains, W.S., Talmey, Gatenby and Christie, similar procedures being carried out on each occasion with equal numbers of controls of the same age. The results are expressed as the number of minimal infecting doses of virus (M.I.D.) withstood by the immunised groups. For instance, if the highest dilution of virus producing lesions in the controls was 10^{-6} , and lesions were produced in the immunised animals at 10^{-2} , but not at 10^{-3} , the group was said to resist 10^4 M.I.D. The method is rough and ready, but fairly satisfactory in practice. We are not satisfied with the use of death and survival as indicators, as much depends on the time the experiment is allowed to continue, the number of mice used and the virulence of the strain.

At the same time the serum from the batch was titrated for antibody against the same four strains, using specific serum from convalescent ferrets as standards. It should be noted that the sera used as standards cannot readily be compared with one another; our Christie standard, for instance, is certainly very poor against Christie virus as compared with W.S. serum against W.S. virus, and the determinations of Christie antibody are correspondingly too high.

The results for intraperitoneal vaccination with formolised virus are given in table I.

TABLE I

Immunity and antibody in mice immunised with formolised W.S. virus

Time since immunisation (days)	Strains of influenza virus used in tests							
	W.S.		Talmey		Gatenby		Christie	
	M.I.D. virus withstood	Antibody in serum	M.I.D. virus withstood	Antibody in serum	M.I.D. virus withstood	Antibody in serum	M.I.D. virus withstood	Antibody in serum
7	100	S/125	0	S/625	0	S/3125	0	S/125
14	$10^3 \cdot 10^4$	S/5	10	S/125	10	S/625	10	S/25
21	$10^3 \cdot 10^4$	S/5	100	S/625	10	S/625	10	S/5
28	$10^3 \cdot 10^4$	S/5	100	S/625	10	S/625	10	S/625
42	10^4	S/5	10	S/125	100	S/625	10	S/625
56	100	S/25	0	S/625	0	S/125	10	S/25

Mice immunised with 2 doses of formolised W.S. virus (10^7 M.I.D. per c.c.) at interval of 1 week

It is clear that there is a very rough correlation between antibody and immunity and that both are better developed to the homologous than to the heterologous strain. As antibody fades so does immunity. In no case can the degree of immunity be regarded as very high, especially as compared with that following intranasal inoculation.

Burnet (1938) has recently reported that vaccination as well as infection with W.S. virus gives rise to a much more generalised immunity than is produced by the use of most other strains; he used living virus and the possibility of localisation of virus in the lung cannot be ruled out.

Immunity after intranasal inoculation is much more complicated. A sample experiment given in fig. 2 makes it clear that there is a

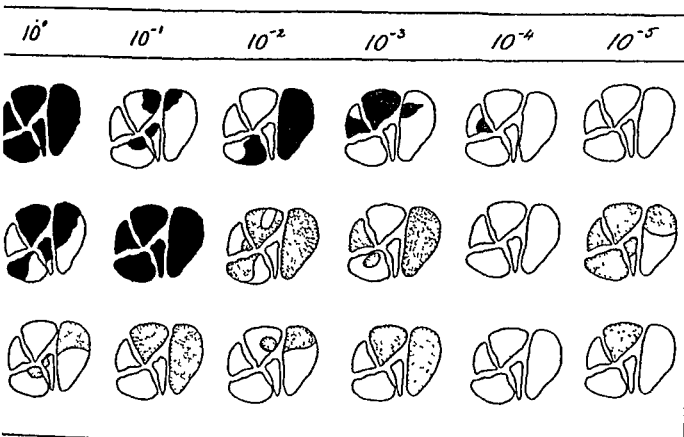


FIG. 2.—Lungs of mice inoculated intranasally with W.S. virus and tested for immunity with Christie virus intranasally 21 days later. Black = recent lesions (Christie); stippled = old lesions (W.S.). The Christie virus used produced lesions in two out of three control mice at 10^{-4} ; no lesions at 10^{-5} .

sharp difference in immunity between animals showing old lesions due to the immunising intranasal inoculum and those showing none. In this experiment a batch of mice was immunised by intranasal inoculation under anaesthetic with 0.05 c.c. of 10^{-6} W.S. virus. Three weeks later a sample of Christie virus was titrated in these immunised animals and in a batch of controls of the same age. When the immunised animals were killed on the sixth day after inoculation their lungs, if they showed anything at all, showed either old grey lesions or recent plum-coloured ones, never both.

In other words, mice surviving intranasal inoculation with W.S. virus with the production of lesions are immune to Christie virus of as high a titre as is available; mice similarly treated without the development of lesions have no advantage over the controls. That this dependence of immunity on the production of lesions is not limited to one strain is shown by the fact that mice inoculated with W.S., with its neurotropic variant (neuroflu) obtained through the kindness of Drs Andrewes and Stuart-Harris, with Talmey or with Gatenby, if they develop lesions, all show complete immunity for at least 2 months to all four strains tested, and usually to a degree far higher than is attained after intraperitoneal inoculation with formolised virus (tables II and III). Mice showing no lesions after intranasal inoculation have no immunity whatever.

TABLE II

Immunity and antibody in mice showing old lesions after intranasal infection with W.S. virus

Time since previous infection (days)	Reaction to different strains of influenza virus							
	W.S.		Talmey		Gatenby		Christie	
	M.I.D. virus withstood	Antibody in serum	M.I.D. virus withstood	Antibody in serum	M.I.D. virus withstood	Antibody in serum	M.I.D. virus withstood	Antibody in serum
7	0	S/3125	0	S/625	0	S/3125	0	S/625
14	>10 ⁵	S/5	>10 ⁵	S/125	>10 ⁵	S/625	>10 ⁴	S/5
21	>10 ⁶	S/5	>10 ⁴	S/125	>10 ⁴	S/125	>10 ⁴	S/5
28	...	S/5	>10 ⁴
56	>10 ⁶	S/5	>10 ³	<S/125	>10 ⁴	c. S/125	>10 ⁵	c. S.

TABLE III

Immunity and antibody in mice showing old lesions following previous infection with influenza virus

Strain used in preliminary infection	Days since first infection	Reaction to different strains of influenza virus							
		W.S.		Talmey		Gatenby		Christie	
		M.I.D. virus withstood	Antibody in serum	M.I.D. virus withstood	Antibody in serum	M.I.D. virus withstood	Antibody in serum	M.I.D. virus withstood	Antibody in serum
Talmey	14	>10 ⁷	S/625	>10 ⁵	S/5	>10 ⁵	S/25	>10 ⁵	S/25
"	28	>10 ⁶	S/125	>10 ⁵	S	>10 ²	S/25	>10 ⁵	S/5
"	56	>10 ⁶	...	>10 ³	...	>10 ⁴	...	>10 ⁵	...
Neuroflu	14	>10 ⁷	S/5-S/25	>10 ⁵	S/625	>10 ⁵	S/125	>10 ⁵	S
"	28	>10 ⁶	S/5	>10 ⁵	S/25	>10 ²	S/25	>10 ⁵	S/5
"	56	>10 ⁶	S/5	>10 ³	S/25	>10 ⁴	S/125	>10 ⁵	S

Andrewes and Smith (1937) record that most of their mice possessing immunity to W.S. after intranasal inoculation showed old lesions in the

lungs; Burnet (1937) states that virus passaged for many generations through eggs, though it produces in mice only minute patches of consolidation in the lungs or no lesions at all, still immunises perfectly and gives rise to high antibody titres. More recently (1938) he has shown that M.E. virus gives much greater immunity against W.S. if old lesions are produced than if they are not. Some strains, however, when inoculated intranasally without producing lesions, yet immunised against the homologous strain. Shope (1935) has also found that a recently isolated strain which produced extensive lesions in mice only after repeated passage would, in its early passages when no evident lesions were produced, immunise mice completely against mouse-lung-adapted virus. This variation between strains needs much further investigation.

The old lung lesions were in some cases exceedingly small, and might have eluded casual observation. It may therefore be suggested that the extensive recent pneumonia in tested animals without obvious old lesions may really conceal minute or even microscopic old pneumonic patches. We have searched carefully for these and think it very improbable that they were present, but this point can be proved only by serial section of the lungs. Notwithstanding Burnet's (1938) statement to the contrary, it is sometimes exceedingly difficult to distinguish old lesions from recent, especially with "weak" viruses (*e.g.* Gatenby). In general, old lesions are grey in colour and deeply sunken beneath the surface; if the whole lobe is involved it is much reduced in size and somewhat granular on the surface. Recent lesions are a deep plum colour; they are sharply separated from the unaffected lung, and if the whole lobe is involved it is usually though not always much enlarged. After infection with "weak" viruses, however, grey "recent" lesions may be produced, and very careful consideration of such matters as collapse and the continuity of general appearance and colour of the lesions may be necessary to determine the point. We have, however, never had a sufficient number of difficult cases to invalidate the general argument; by about 2 months after infection the difficulty hardly ever arises.

Although animals possessing no immunity have no antibody, there appears to be no correlation between immunity and antibody in animals showing old lesions. Indeed the antibody response to intranasal inoculation with development of lesions appears much the same as that to vaccination with formolised virus, though the immunity produced is much more striking and entirely different in its lack of strain specificity.

The interference phenomenon

As a result of extensive experiments Rickard and Francis feel that antibody is insufficient to account for the immunity developed after intraperitoneal inoculation with living virus. They argue as follows. Mice inoculated intraperitoneally with large doses of living virus show after about 24-48 hours quantities of virus in the lungs sufficient to kill large

numbers of mice if inoculated intranasally under anæsthetic. Notwithstanding this, such mice do not die, though small lesions may be produced, but become in time solidly immune. Indeed they show considerable immunity to intranasal inoculation with homologous virus as early as two days after intraperitoneal vaccination, although there is no demonstrable circulating antibody. They suggest that virus accumulated in the lung from the intraperitoneal inoculation interferes in some way with the entrance of virus inoculated intranasally, and thereby reduces the degree of infection.

With their view that virus can be recovered in large quantity from the lungs of mice inoculated intraperitoneally with sufficiently large amounts of living virus we are in entire agreement; but though we have repeated their experiments on the immunity developed thereafter on several occasions on a large scale with W.S. and P.R.8 strains, we have been entirely unable to confirm their finding that immunity develops before antibody is demonstrable in the circulation.

In fact up to the 4th day after intraperitoneal inoculation the lesions produced by intranasal infection in vaccinated animals at all dilutions were more extensive and deaths were more frequent than in the controls (fig. 3). No doubt the lesions at the very high dilutions were due to the vaccinating dose of virus. By the fifth day traces of antibody could be demonstrated in the circulation and slight differences in favour of the vaccinated animals could be made out.

We communicated our findings to Dr Francis who very kindly sent us the following suggestions.

(1) Our immunising dose was too small. As Rickard and Francis use the M.L.D. as their unit and we prefer the M.I.D. as less affected by the time of duration of the experiment and the virulence of the virus, it is difficult to compare values. Unfortunately following our usual practice we killed all survivors on the sixth day after inoculation, while Rickard and Francis allowed their experiments to continue for ten days. In a recent experiment with W.S. virus we have determined the effect on the estimation of the number of M.L.D. per c.c. of a sample produced by varying the duration of the experiment from six to nine days. On the sixth day the titre was 10^4 M.L.D., on the 7th day 10^5 , on the eight and ninth 10^6 ; the M.I.D. per c.c. remained the same at 10^8 . In other words by waiting a few more days it is possible to increase the estimate of the number of M.L.D. of a virus sample a hundredfold, while the M.I.D. remains unchanged. We think this explains the difference between us and Rickard and Francis, whose M.L.D. titre (up to the tenth day) was 10^6 while ours at the sixth day is only $10^3 \cdot 10^4$.

In any event our immunising dose produces lesions in the lungs of about half the mice injected and if the views of Rickard and Francis on the amount of virus which it is necessary to inject intraperitoneally to produce these lesions are correct, the titre of our virus cannot be much lower than theirs.

(2) Our results, especially the somewhat irregular distribution of lesions in the controls, might be due to secondary bacterial infection. To avoid this we have throughout used virus filtered through gradocol membranes capable of retaining bacteria ($0.6-0.8 \mu$). On the one occasion when unfiltered P.R.8 virus was used for vaccination to duplicate the experiments of Rickard and Francis more accurately, large numbers of mice died in 24 hours with

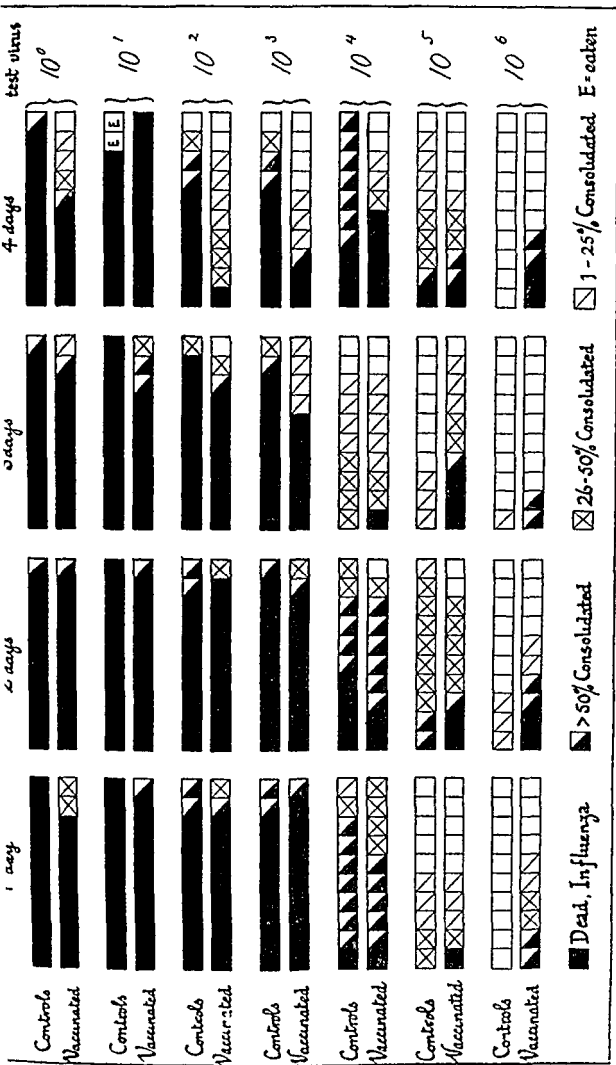


FIG. 3.—Immunity against intranasal infection with varying dilutions of WS virus at various times after intraperitoneal inoculation with one dose of 0.5 cc of living WS virus (10^0 M.I.D. per cc). Each square represents a mouse. The results for the fifth day, which are more favourable to the vaccinated animals, have been omitted, to save overcrowding. E = eaten by other mice.

lesions in no way resembling influenza and the survivors showed exactly the same distribution of immunity as mice inoculated with filtered virus.

(3) It is still necessary to explain why mice inoculated intraperitoneally with large amounts of living virus do not die although they show two days thereafter an amount of virus in the lung which would kill many thousands of mice if inoculated intranasally.

It appeared to us possible that the development of lesions in the lung might not be an essential sequel to the multiplication of influenza virus in them. We therefore inoculated batches of mice with falling tenfold dilutions of filtered influenza virus intranasally under anæsthetic. Half of these mice were killed at 2 days; the rest were allowed to survive for ten days to determine the highest dilution of virus producing lesions in the lungs. The lungs of those killed at 2 days were ground up with quartz and broth-saline and centrifuged. Falling tenfold dilutions of the supernatant fluid were inoculated intranasally under anæsthetic into small mice.

It is clear from table IV that mixed lungs from mice after intranasal infection with a dose of W.S. virus which produces minimal lesions or none at all may contain at two days quantities of virus capable of producing extensive lesions or even of killing large numbers of mice. We have also frequently observed that mice inoculated with lungs from mice killed two days after infection die sooner than those of the infected batch allowed to survive.

TABLE IV

Virus in lungs (mixed) 2 days after intranasal inoculation of W.S. virus

Dilution of virus inoculated	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-8}	10^{-7}
Effect in mice not killed at 2 days	D	D	L	L	L or O	O
Titre of virus in lung (M.L.D.) at 2 days	10^6	10^5	10^4	10^4	10^3	0
Titre of virus in lung (M.I.D.) at 2 days	10^8	10^9	10^8	10^6	10^5	? 10

D = died before 10th day; typical influenzal lesions

L = killed on 10th day; " " "

O = " " " " ; no lesions

Twelve mice were inoculated intranasally with 10^{-5} W.S. virus. At two days 6 were killed and their lungs collected. The rest died in about 8 days from influenza. Suspensions of lungs of those killed on the second day were inoculated intranasally under anæsthetic into mice; most of these died on the 3rd or 4th day, i.e. 2-3 days before the death of these allowed to survive in the original group. Smorodintseff and Ostrovskaya (1937) have obtained results much more striking than ours; lungs of mice inoculated with virus diluted to 10^{-12} , though themselves showing no lesions, would after repeated passage produce typical influenza in mice. We think it likely that the difference is due to the high degree of mouse adaptation of our viruses, for Stuart-Harris *et al.* have shown that recently isolated strains can be propagated for several generations in mice before producing lesions. Burnet's results (see p. 45) support this view.

We have also determined the titre of influenza virus in individual lungs of mice two days after intranasal infection. The results (table V) show that the lungs can be divided sharply into two

TABLE V

Titre of influenza virus in individual lungs 2 days after intranasal infection with 10^{-7} WS virus

Mouse lung no	1	2	3	4	5	6	7	8	9	10
Virus titre, M L D (10 days)	0	10^5	10^6	0	0	0	0	0	0	10^5
Virus titre, M I D (10 days)	0	10^5	10^7	0	0	0	0	0	0	10^6

Of 28 mice of same group allowed to survive 10 days, 13 developed lung lesions

groups, those showing large quantities of virus and those showing none. Since the lungs of those mice killed at ten days can similarly be divided into two groups, those showing lesions and those showing none, we think it likely that those showing large quantities of virus at 2 days would have developed lesions, while those with no virus would not have done so if allowed to survive for 10 days.

In order to prove that the lesions and deaths were due to influenza virus, a matter of considerable importance where unfiltered virus is used, influenzal antibody was mixed with some of the dilutions of two day lungs and inoculated intranasally into mice under anaesthetic. None died and very few developed lesions, though at the corresponding dilutions of virus alone almost all the mice had died. There seems no doubt that in the main the deaths and lesions were due to influenza virus, and we are left with the paradox that two days after intranasal inoculation of mice with high dilutions of influenza virus the lungs may contain virus sufficient to kill thousands of mice, and yet at ten days they show only minimal lesions.

It is we think probable that when only a few virus particles are inoculated, each can proliferate locally to about 10^6 particles before the mass of virus becomes greater in volume than the cell in which it is parasitic. Consequently, though large quantities of virus may be present, they may be strictly localised to a few cells in the lung, and only negligible lesions be produced. Certainly in this case the interference phenomenon cannot be invoked.

If this is so, antibody will explain the immunity developed after intraperitoneal inoculation with living virus without any further mechanisms. The remarkable spread of immunity to heterologous strains after intranasal infection is as yet unexplained, virus neutralising antibody is certainly insufficient and we have as yet no information on complement fixing antibody in mice.

Lastly, in the light of our experiments and those of Rickard and Francis on the development of lesions in the lung after intraperitoneal inoculation with living virus in sufficient amount, it is interesting to speculate on the strain immunity developed by such animals. Will it be the same whether lesions are developed or not? Or, if lesions are developed, will the immunity spread to heterologous strains? Possibly the somewhat divergent results obtained after immunisation with living virus may find their solution here.

Summary

1. After intranasal inoculation with living virus or intraperitoneal vaccination with two doses of living or formolised virus, mice develop both antibody and immunity to influenza of varying quality and degree.

2. The rise in antibody is fastest in the animals given two doses of living virus intraperitoneally; all three groups reach the same maximum two weeks after the first inoculation. In some mice infected intranasally antibody has disappeared by six weeks; in others it may remain more than 3 months. Antibody persists about 8 weeks in animals vaccinated with two doses of formolised virus, 12 weeks in those vaccinated with living virus. Mice vaccinated with one dose of living virus intraperitoneally show very poor antibody levels.

3. In mice vaccinated intraperitoneally with formolised virus antibody titre and immunity to the main strains run parallel. Animals infected intranasally, though their antibody shows no striking difference from those vaccinated with formolised virus, are immune to all four strains.

4. After intranasal inoculation with W.S., its neurotropic variant, Talmey and Gatenby, the production of lesions is essential for the development of antibody and immunity.

5. Circulating antibody can account for all the characters of the immunity to the homologous strain possessed by animals vaccinated intraperitoneally with living virus.

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THE OCCURRENCE OF INTRANUCLEAR INCLUSIONS IN CULTURES OF FŒTAL LEPTOMENINGES

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(PLATE X)

THE occurrence of acidophilic intranuclear inclusion bodies in human gliomata has been reported by one of us (Russell, 1932) and confirmed by Wolf and Orton (1933 34) who also found similar bodies in examples of meningioma, perineurial fibroblastoma and hemangioblastoma. The appearance of these bodies in living and in fixed and stained cultures of meningiomata has been described recently by Bland and Russell (1938) although optically empty by transmitted light in the living state, they contain, as shown by photographs using ultra violet light, a granular content the nature of which is unknown. In Mr J E Barnard's opinion (Bland and Russell, *loc cit* p 299) the appearances shown in such photographs do not suggest that the body contains a virus, although this interpretation is not regarded by him as conclusive.

Acidophilic intranuclear inclusion bodies are known to be associated with certain virus diseases. They form a conspicuous feature also in the renal carcinoma of *Rana pipiens*, which has been studied by Lucké and has recently been shown by him (1938) to be due in all probability to a virus. It appeared therefore at least possible that the inclusion bodies present in human tumours might also be due to a virus. In view of the known species specificity of many viruses—and hence the necessity of using human tissues for experiment—we undertook to find out whether inclusion bodies could be artificially produced in cultures of healthy human foetal tissues by the addition to the culture medium of extracts of suitable human tumours.

Foetal leptomeninges were chosen for our experiments both because they are readily grown *in vitro* and because adequate supplies of fresh tissue from meningeal tumours were obtainable from the neurosurgical clinic of this hospital.

* Working with a grant from the British Empire Cancer Campaign

† Working for the Medical Research Council

Technique

The leptomeninges were taken from fresh sterile fetuses obtained by laparotomy and were usually cultured within 48 hours. On two occasions the material was kept for 3 and 6 days respectively at about 4° C. Cultures from this grew satisfactorily but a longer period than usual elapsed before migration of cells began.

Part of the tissue was used for wet film preparations which were stained by Mann's method (Russell, Kraysenbühl and Cairns, 1937) and examined for the presence of intranuclear inclusion bodies.

The hanging drop technique was used for the cultures. The medium consisted of 1 part of human plasma, 1 part of fowl plasma and 2 parts of chick embryo extract. It was found advisable to increase the concentration of sodium chloride in the Pannet and Compton's solution to 0.8 per cent. The first subcultures were made after 3 days and all others after 4-5 days. All the cultures were fixed and stained as whole mounts. As a rule they were fixed for 10 minutes in acetic-alcohol, washed for 1½ hours in several changes of distilled water and stained with dilute Heidenhain's hæmatoxylin or with Mayer's hæmatoxylin. Other fixatives and stains were used as shown in the table. In general, fixatives other than acetic-alcohol or saline-formaldehyde result in the obscuration of the zone of outgrowth by precipitate in the medium. Such a precipitate can, however, be avoided as suggested by Glücksmann (personal communication) by making a small hole in the medium near the explant before fixation; a fine jet of warm saline and then fixative is squirted round the explant until the zone of outgrowth has become transparent. Further fixation and staining can then be carried out in the usual way. It was found that the times required for staining the preparations were one-third to one-half as long as for staining sections.

Material and results

Leptomeninges from 27 fetuses ranging in age from 8 to 28 weeks were cultured for varying lengths of time. Microscopic examination of stained cultures revealed intranuclear inclusion bodies in the migrating cells from 48 hours onwards up to 31 days, when culture was discontinued. The inclusion bodies were seen in cultures from 25 of the 27 fetuses and in approximately one-third of the cultures examined. They were generally scarce, between one and eight being found in a zone of outgrowth containing several hundred cells. Rarely as many as 30-40 inclusions were found in a single culture. They were seldom observed within cells forming the explant but were usually found in the zone of outgrowth and were most conspicuous when the nuclei were enlarged and flattened (fig. 1). Most of the inclusion bodies were spherical and were surrounded by a clear halo outside which was a ring of nucleoplasm. Their contents were finely granular and gave staining reactions similar to those of the cytoplasm (table). Special stains for fat, glycogen and nucleic acids were negative. The nuclei in which inclusions were found appeared otherwise normal and were evidently capable of division, since in one example a nucleus was identified in metaphase (fig. 2). The inclusion body here is clearly visible in the cytoplasm near the chromosomes.

INTRANUCLEAR INCLUSIONS IN CULTURES



FIG. 1—Human leptomeningeal cell in culture showing intranuclear inclusion. Note granular content. Heidenhain's hematoxylin $\times 850$.



FIG. 2—Nucleus in metaphase in culture of human leptomeninges with inclusion body lying free in cytoplasm. Heidenhain's hematoxylin $\times 850$.

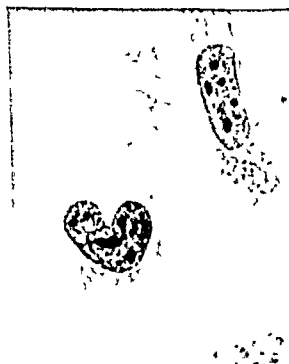


FIG. 3—Deeply indented nucleus in culture of human leptomeninges. Heidenhain's hematoxylin $\times 850$.



FIG. 4—Cells from culture of human leptomeninges showing (A) nucleus almost completely enveloping a portion of cytoplasm, (B) nucleus with inclusion. Heidenhain's hematoxylin $\times 850$.

Thus, under the conditions of our experiments, intranuclear inclusion bodies appeared spontaneously in cultures of human foetal leptomeninges without the deliberate addition of tumour extract or of any other stimulating substance. It therefore appeared improbable that a virus could have been implicated in the formation

TABLE
Staining reactions of intranuclear inclusions in cultures of foetal leptomeninges

Fixative	Stain	Nucleoplasm	Chromatin and nucleolus	Inclusion body	Cytoplasm
Acetic alcohol	Heidenhain's hæmatoxylin	Grey	Black	Grey	Grey
Carnoy's fluid	Hæmatoxylin and erythrosin	Pale blue	Deep blue	Lilac	Lilac
Methyl alcohol	Giemsa	Mauve with purple granules	Deep purplish-blue	Pale blue	Pale blue
Mercuric chloride and formaldehyde	Laidlaw's acid fuchsin orange G with Weigert's iron hæmatoxylin	Grey	Grey-black	Light grey	Light grey
Zenker's fluid	Teulgen's stain for nucleic acids	Grey	Deep violet, larger nucleoli with green centres	Pale green	Pale green
Boun (Allen)	Best's method for glycogen	Light grey	Dark grey	Pinkish-grey	Pinkish grey
Saline formaldehyde	Bleu B Z L for fat	Pink	Deeper pink	Pale pink	Pale pink
Flemming	Osmic acid for fat; carmalum counterstain	Faint pink	Deeper pink	Faint pink	Faint pink

of these bodies. But in view of their known association with virus infections it seemed necessary to consider the possibility that the conditions of our experiments had permitted the entry of a virus. Such a virus might have been derived: (1) from the laboratory environment, (2) from the media used in culture, or (3) from the tissues themselves.

(1) Further experiments were made at the Strangeways Research Laboratory, Cambridge, by kind permission of the Director, Dr Honor B. Fell. The laboratory has an isolated position in open country; moreover no research work on viruses was in progress there at the time of the experiments. Nevertheless intranuclear inclusion bodies appeared in cultures with approximately the same

frequency as before. They were also observed in cultures of human foetal leptomeninges independently prepared by Dr J. O. W. Bland at St Bartholomew's Hospital, to whom our thanks are due for allowing us to examine his preparations.

(2) A virus might be introduced with samples of human plasma, fowl plasma or chick embryo extract. The known species-specificity of viruses minimises the likelihood that the two latter vehicles were responsible. Different samples of human plasma from healthy adults were used, but without significant effect upon the cultures. Finally human plasma was entirely replaced by fowl plasma in preparing the medium. Healthy cultures were obtained by using 1 part of fowl plasma to from 4 to 8 parts of embryo extract. The liquefaction about the explants in such cultures was, however, inadequate and the Maximow technique was therefore adopted, the explants being washed at 48-hourly intervals for 10 minutes with Pannet and Compton's solution followed by a wash with chick embryo extract for 5 minutes. Intranuclear inclusion bodies were found in the cultures as before.

(3) The possibility that a virus is carried by the foetal tissues themselves cannot be dismissed. If this is so the distribution of virus must be widespread, if not universal, in such tissues. Examination of the stained films prepared from the fresh foetal leptomeninges at the time of culture disclosed no typical inclusion bodies. In a few films an occasional nucleus contained a vacuole which was too small to be detected except with a $\frac{1}{12}$ inch objective. The possibility that these represent an early stage of inclusion formation cannot be dismissed, although they appeared more rarely than did the inclusion bodies in cultures.

Further experiments showed that similar intranuclear inclusion bodies appeared in cultures from the leptomeninges of embryo rats and chickens and in fibroblast cultures from the lungs of human and chick foetuses. The phenomenon therefore is neither peculiar to the leptomeningeal cells nor to the human species.

Finally attempts were made to influence the appearance of inclusion bodies in cultures of human foetal leptomeninges by altering (a) the pH of the medium, ranging from 6.6 to 7.6, (b) the tension of the medium by the addition of bile, (c) the sodium chloride concentration of the medium and (d) the temperature of incubation. None of these modifications had any effect upon the appearance of the inclusion bodies in the cultures.

Discussion

Our experiments have shown that intranuclear inclusion bodies appear spontaneously in cultures of foetal leptomeninges. Although we have been unable to influence or explain their appearance, the likelihood that they are attributable to a virus seems remote.

Uncertainty as to the significance of these bodies has increased within the last few years. In 1935 it was widely accepted that their presence was indicative of virus activity, although in that year Cowdry, while admitting this association, held that they should not be regarded as pathognomic of such activity in the absence of experimental proof. The rather wide distribution of these bodies in the otherwise healthy tissues of various animals (Cowdry, Lucas and Fox, 1935) and in the submaxillary glands of young infants (Farber and Wollbach, 1932) suggests that this cautious attitude is well founded. Yet the demonstration of a virus of low pathogenicity in the salivary gland disease of guinea-pigs and in mice showing inclusions in the liver cells (Findlay, 1932) supports the view that a saprophytic virus may well be responsible for similar appearances in other tissues and in other species. On the other hand, Blackman (1936) observed intranuclear inclusions in the kidneys and liver of children dying from lead poisoning. Olitsky and Harford (1937) produced similar inclusions in guinea-pigs and rabbits by the subcutaneous injection of various organic and inorganic substances, notably aluminium hydroxide. The inclusions were produced within mononuclear and giant cells at the site of inoculation. The similarity of the inclusion bodies to those associated with virus infections was close, but no virus could be demonstrated experimentally in the affected tissues. These inclusion bodies may therefore be ascribed to a chemical or physical change within the nucleus. The possibility that they may be produced as a result of functional activity has been suggested by Gilmour (1937), who observed the constant appearance of inclusions in the human male genital tract with the onset of spermatogenesis. Again acidophilic inclusions in both nuclei and cytoplasm were found by D'Aunoy and Evans (1937) in the normal chorio-allantoic membrane of the developing chick embryo.

The possibility that the intranuclear inclusion bodies appearing in our cultures were of cytoplasmic origin is suggested by their identical staining properties and the frequent presence of deep indentations or bays within the nuclear membrane, causing the nucleus to appear sometimes as an incomplete ring (figs. 3 and 4). By a continuation of this process a portion of cytoplasm might be nipped off and enclosed within the nucleus. This possibility deserves consideration in view of the presence of melanin pigment in otherwise similar inclusion bodies in melanotic tumours (Ludford, 1924). Melanotic tumours have, however, been examined by Apitz (1937), who traced the development of the inclusions from small vacuoles which became larger and ultimately led to the death of the cell. Although melanin pigment was present in the larger forms he rejected the idea that they were of cytoplasmic origin because at certain stages the pigment was present in the inclusion body while

absent from the cytoplasm. He suggested that the vacuoles arose within the nucleus through retention of a physiological secretion, the nucleus being regarded as responsible for the production of melanin.

Our observation of an apparently free inclusion body in the cytoplasm of a cell undergoing mitosis is more suggestive of a nuclear than of a cytoplasmic origin. If this is correct then the inclusion bodies in our cultures may have arisen from the minute "vacuoles" occasionally seen in direct films of the foetal leptomeninges, although phases in this transition were not demonstrated. Such a sequence was, however, claimed by Benninghoff (1923) in the formation of the "Lochkerne" observed by him in fixed preparations of connective tissues from many species including man, and later by Chlopin (1931-32) in cultures of human embryonic mesenchyme. Neither of these authors demonstrated the granular contents of the intranuclear bodies they described but, from their illustrations, we feel confident that the structures they observed were essentially similar to ours.

These considerations make it difficult to accept the view that virus activity is responsible for the appearance of acidophilic intranuclear inclusions as a whole. While some are clearly associated with a virus infection and may even be composed of virus particles it appears that others are more probably the result of a physical or chemical change within the nucleus. The nature of the intranuclear bodies in human tumours remains uncertain.

Summary

1. Acidophilic intranuclear inclusion bodies appeared spontaneously in cultures of human foetal leptomeninges although absent from the tissues from which the cultures were made.

2. They also developed in cultures of leptomeninges from chick and rat embryos and in fibroblast cultures from the lungs of both human and rat foetuses.

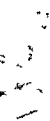
3. Attempts to influence the development of the inclusion bodies by modifying the media used for culture and by altering the environment of the experiments were unsuccessful.

4. No evidence has been found in support of the theory that a virus is responsible for the appearance of these bodies.

Our thanks are due to Dr H. B. Fell for allowing the work to be repeated at the Strangeways Research Laboratory, Cambridge, and to the consulting obstetricians of the following hospitals for their kindness in supplying us with material: the London, St Bartholomew's, King's College, St George's, the Royal Free, St Mary's, the Westminster, and the British Postgraduate Medical School.

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CONGENITAL DIVERTICULUM OF THE LEFT VENTRICLE OF THE HEART IN A CASE OF EPILOIA

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(PLATES XI-XIV)

It has long been recognised that tuberose sclerosis of the brain is frequently associated with tumour-like abnormalities in other organs, notably the skin, kidneys and heart. The name epiloia, first suggested by Sherlock (1911), has been applied by clinicians to the syndrome of epilepsy, mental defect and sebaceous adenoma, although it is sufficiently realised that this triad constitutes only one well defined sub-group of the clinical variants displayed by a protean disease-complex which still has no distinctive name. According to Critchley and Earl (1932) tuberose sclerosis of the brain is probably the most constant feature of the disorder, but it is by no means an invariable concomitant.

Abnormalities of the heart found in cases coming to post-mortem almost always take the form of rhabdomyomata, small single or multiple tumours of striped muscle tissue, usually incorporated in the ventricular walls. Cardiac abnormalities other than rhabdomyoma are extremely rare and in the few cases described have consisted of stenoses or other malformations limited to the region of the truncus arteriosus. In an otherwise typical case of epiloia recently investigated at Stoke Park Colony we have encountered what we believe to be a unique anomaly of differentiation of the heart, in the shape of a large heterotopia of aortic tissue lining a congenital diverticulum of the left ventricle. The association of this rare tissue malformation with the otherwise characteristic cerebral and visceral features of the disease provides strong support for the view that the lesions of epiloia are mere anomalies of differentiation and are not intrinsically neoplastic, tumour-like though their appearance may be.

* Working under the auspices of the Burden Mental Research Trust.

Clinical history

The patient, a boy of twelve, was admitted to Stoke Park Colony as a case of severe mental defect with epilepsy. He was the eldest of three children one of whom had been drowned in the bath, apparently at his hands. He had walked at one year and began to talk at four years, but his speech was always indistinct and often unintelligible. On admission he was classed as an imbecile of mental age less than three years. On examination he showed the typical facial lesions of adenoma sebaceum with patches of "shagreen" over the trunk and small papillomatous skin tumours on the back and chest. Apart from signs of mild pyramidal impairment on the right side the central nervous system was normal. The epileptic fits, which averaged twice weekly on admission, gradually increased in frequency and severity and latterly occurred several times a day. There was progressive mental deterioration and death finally occurred from bronchopneumonia at the age of twenty-one years. In view of the autopsy findings it is of interest to record that clinically no cardiac lesion was detected by the medical officers of the Colony at repeated examinations, although a note on his admission papers stated that there was at one time a soft apical systolic murmur.

Post-mortem examination

Anatomical diagnosis. Acute bronchopneumonia of right lung following bronchiectasis; suppurative softening with abscess formation; acute fibrino-purulent pleurisy. Lesions of epiloia.

The post-mortem findings relevant to the present discussion were as follows.

Heart

Macroscopic. Slightly enlarged, right chambers somewhat dilated. Myocardium firm, valves healthy. Section through the left ventricle revealed a gross structural deformity which had been entirely unsuspected on naked-eye inspection. A large diverticulum with several ramifications occupied a considerable part of the ventricular muscle, interrupting the normal pattern of the columnæ carneæ and greatly diminishing the total quantity of contractile tissue (fig. 1). The remainder of the ventricular cavity was lined by normal endocardium; at the mouth of the diverticulum this gave place to a tough resistant layer of considerable thickness which covered the inner surface of the diverticulum, becoming attenuated in its deeper recesses. Section through the ventricle exposed several of these ramifications, all lined by the same tough membrane which offered considerable resistance to the knife, cutting in precisely the same manner as aortic tissue although separated by a wide interval from the aorta above. Surrounding the diverticulum and embedded in the substance of the myocardium was an irregular mass of adipose tissue which at one place abutted on the epicardium, here merging with the normal sub-epicardial fat. No discrete tumours suggestive of rhabdomyoma were found in any part of the cardiac muscle.

Microscopic The impression gained on naked eye inspection was that we were dealing with a heterotopia of aortic tissue, and histologically this view was amply confirmed. Sections through the wall of the diverticulum showed dense musculo elastic tissue in which the abundant elastic fibrils were well demonstrated by orcein and by Weigert's elastic tissue stain (fig 2). These fibrils diminished in number as the wall became attenuated, but were still recognisable in the deepest recesses. In parts near the mouth of the diverticulum the structure was identical with that of the aorta and the total thickness only slightly less.

Intervening between the diverticulum and the muscular wall of the ventricle was an irregular mass of adipose tissue, microscopically showing a very meagre stroma except in the parts adjacent to the diverticulum, where a denser fibro-cellular element was contributed by the aortic adventitia. A remarkable feature of this stroma was the presence of scattered cells of large size, up to $120\ \mu$ in diameter (fig 3). The majority of these cells possessed a single compact nucleus, usually centrally placed, a few contained two or at most three similar nuclei. Most of the cells had abundant cytoplasm containing a densely packed mass of coarse eosinophile granulations and possessed a thick cell membrane which appeared to form no contacts with the surrounding stroma, a few showed fine fibrils of variable length which radiated into the surrounding tissues. These peculiar cells closely resembled the primitive muscle cells of rhabdomyoma, and although no striations were observed in the associated fibrils where present, we have concluded that they represent this primitive type of structure. We have recently had the opportunity of examining a typical cardiac rhabdomyoma found at autopsy in another Colony inmate, and a comparison with the cells composing this characteristic tumour leaves little doubt as to the identity of those now described.

The underlying myocardium in the numerous sections examined was of normal histological type throughout, and no nodules suggestive of frank rhabdomyoma were discovered.

Brain

Macroscopic The brain was within normal limits of size, weight and convolutional patterning, but its surface presented numerous sclerotic areas scattered over both hemispheres, fourteen were visible on the left side and twenty on the right. These areas were limited to the grey matter of the cortex, the white matter on section showing no naked eye abnormality. Photographs of this brain appear in *A cerebral atlas*, recently published from Stoke Park Colony (Berry, 1938). Characteristic "candle gutterings" were found in the walls of the lateral ventricles, and in addition

three discrete sub-ependymal tumours about $\frac{1}{4}$ in. in diameter were present, one attached to each optic thalamus, the third to the right caudate nucleus.

Microscopic. The *sclerotic areas* in the cerebral cortex showed all the typical features of tuberosc sclerosis: reduction in number and marked disorientation of the nerve cells, increased numbers of astrocytes, a heavy fibrillary gliosis and many characteristic giant cells of bizarre form (fig. 4). These last commonly appeared in compact groups, but single examples were frequently seen scattered through the affected areas and also, more rarely, as heterotopias in the molecular layer and sub-cortical white matter. Most of the giant cells appeared to be glial in type, but in Bielschowsky preparations some abnormal nerve-cell derivatives could be distinguished by their neuro-fibrillary contents.

Sections taken from *macroscopically normal gyri* showed that the characteristic lesions were much more widespread than had at first appeared. Abnormal cells of spongioblastic type and isolated giant cells were frequently found in these apparently unaffected areas (fig. 5), bearing witness to the generalised nature of the abnormal process at work. Similar changes were also present in the thalamus (fig. 6), but the cerebellum appeared to be unaffected.

In the *sub-ependymal nodules* two distinct structural types were encountered. The first was best seen in the thalamic tumours, which consisted of dense whorls of fibrillary neuroglia embracing groups of large bipolar cells of giant size (figs. 7 and 8); at places these cells were seen to contribute a lash of fibres to the glial meshwork of the tumour. The two thalamic nodules showed a variable vascularity and one of them contained massive calcium deposits. The second histological type was found in the ependymal nodule attached to the right caudate nucleus. This was richly cellular, composed of round or oval cells lying in a very delicate glial stroma (fig. 9). The nuclei were oval in shape and contained uniformly distributed chromatin granules resembling those of ependymal cells; the cell outlines were usually ill-defined and the cytoplasm was scanty, but here and there contained prominent deeply stained granules of regular size identical in appearance with the blepharoplasten found in the cells of ependymomas (figs. 10 and 11). In this tumour also large calcium deposits were present.

Kidneys

Macroscopic. Moderately enlarged, the left more than the right. Both showed numerous projections of tumour-like tissue scattered irregularly over the surfaces. The capsules stripped readily, revealing many more smaller tumours embedded in the renal cortex, but for the most part lying flush with its surface. On

CONGENITAL DIVERTICULUM OF HEART

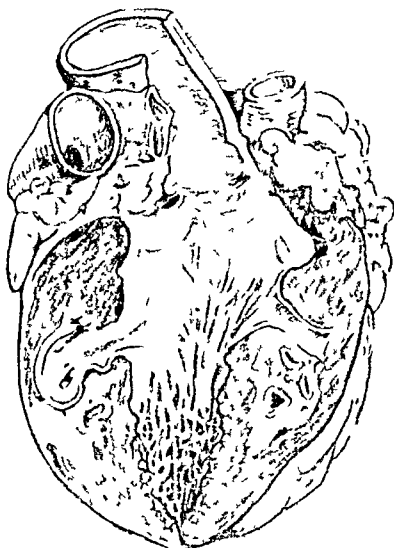


FIG. 1.—Section of left ventricle, showing the large ramifying diverticulum occupying the muscular wall. Slightly reduced.



FIG. 2.—Low power view of wall of diverticulum showing a musculo-elastic structure indistinguishable from that of the aorta. Weigert's elastic stain. $\times 60$.

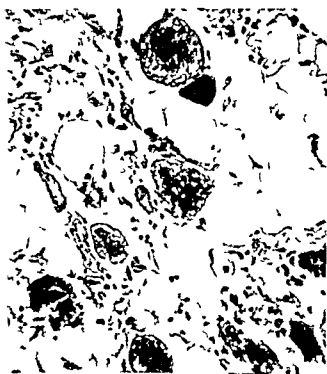


FIG. 3.—Heterotopic fatty tissue adjoining the diverticulum and containing scattered primitive muscle cells. H and E. $\times 150$.

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CONGENITAL DYSPLASIA OF BRAIN

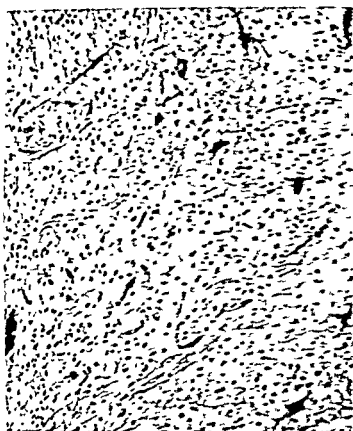
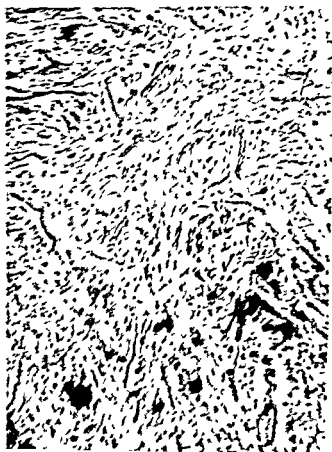


FIG. 4—Abnormal glial forms in a sclerotic area of the cerebral cortex. D-121-H method for astrocytes. $\times 100$.



FIG. 5—Abnormal glial cells in a sclerotic area of cortex. Ramon y Cajal's gold sublimate method. $\times 250$.



CONGENITAL DIVERTICULUM OF HEART



FIG. 8—Giant bipolar cells in thalamic tumour
Ramon y Cajal's gold sublimate method
 $\times 600$



FIG. 9—Sub-ependymal tumour attached to
caudate nucleus—Tannin silver method
Del Río Hortega's 4th variant $\times 100$



FIG. 10



FIG. 11

FIGS. 10 and 11—High power view of cells in fig. 9, showing granules resembling
blepharoplasts $\times 1000$ and $\times 800$

100

~ CONGENITAL DIVERTICULUM OF HEART



FIG. 12.—Section of one kidney showing tumour-like masses occupying mainly the cortex. $\times \frac{1}{2}$.

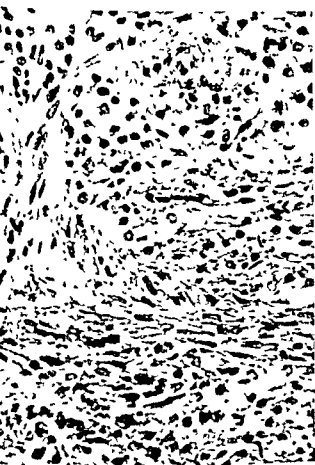


FIG. 13.—Ill-defined mesenchymal bundles composing the renal tumours. H. and E. $\times 270$.

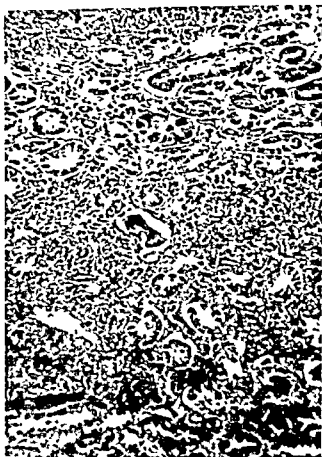


FIG. 14.—Renal cortex, showing tubules of normal contour embedded in mesenchymal tissue. H. and E. $\times 100$.

(Rehder, 1914; Schuster, 1914; Critchley and Earl, 1932)—but we have been unable to find any previous record of a ventricular malformation such as is here described.

In our view the present case is of peculiar interest in affording cogent evidence for the purely dysontogenetic nature of the pathological syndrome under discussion. Much of the controversy which exists appears to have arisen from a preoccupation on the part of investigators with the changes occurring in the nervous system. Bielschowsky (1923-24) maintained that the glial overgrowth is neoplastic and regarded the neural abnormalities commonly associated with it, such as giant nerve cells, disturbed architectonics and naked axis cylinders, as secondary to the gliomatosis. On the other hand the opinion of earlier workers (Alzheimer, 1904; Vogt, 1908) that the essential feature of the disease is a primary malformation of the nervous system as a whole has certainly the greater weight of evidence in its favour (Globus, 1932). This school also has stressed the so-called blastomatous features of the condition, probably under the influence of the Cohnheim theory of embryonic rests stimulated to neoplastic growth by local mechanical disturbances. Yet in the present case we have been impressed by the remarkably orderly appearance of the aberrant tissues in whatever part of the body they are found. While it must be admitted that the microscopic picture presented by the glial mass in fig. 7 resembles somewhat that of genuine neoplasia, even here evidence of progressive growth is lacking; mitoses were not observed and the adjacent parts of the basal ganglia showed no sign of infiltration or of pressure distortion. In the cerebral cortex the sclerosed areas lie side by side with grey matter apparently normal except for minor glial abnormalities, and the scattered heterotopic cells which we have found occasionally in the sub-cortical white matter bear no sign of blastomatous growth.

In the kidneys this "symbiosis" of functioning and aberrant tissue is specially striking; although there has clearly been an excessive formation of mesenchymal tissue there is nowhere any sign of progressive growth and the adjoining glomeruli and tubules show not the slightest degree of distortion or pressure. A further interesting feature is the frequent occurrence of fatty tissue as a component part of the renal tumours, as well as its appearance in quantity surrounding the wall of the cardiac diverticulum. A similar incorporation of adipose tissue has previously been noted in gastric adenomyoma and in heterotopic pancreatic tumours (Taylor, 1927) and appears to be not uncommon in congenital malformations. In another case of tuberosc sclerosis recently investigated here, several massive renal tumours were composed almost entirely of fat, so that the whole organs actually floated in water. Here again there was no microscopic evidence of recent

growth or of interference with the adjacent functioning tissues and, as also in the present case, no clinical symptoms referable to the kidney were complained of during life. When it is recalled that the fatty tissue surrounding the cardiac diverticulum contained cells indistinguishable from embryonic muscle cells, it seems clear that these also owe their origin to the same error of differentiation which in neighbouring foetal tissue has produced the major deformity of the ventricle. The association of these cells with an obvious malformation can be no mere coincidence, and suggests with a high degree of probability that cardiac rhabdomyomata are themselves of a similar dysontogenetic nature. Farber (1931), in a comprehensive investigation of these tumours, concludes (p. 127): "The evidence seems to point against a true neoplastic nature of this growth. . . . To put it into the hamartoma grouping and to leave its origin cloaked in the obscurity characteristic of so many deviations from the normal in growth, seems to dispose best of all this perplexing question in the rhabdomyoma literature." In our opinion this conclusion would apply with equal force to all the tissue disturbances encountered in the present case.

There are strong grounds therefore for the belief that all the lesions of epiloia are due to errors of differentiation occurring early in foetal life. The ultimate mechanism is obscure, for it cannot reasonably be sought in local mechanical disturbances affecting the many diverse and distant organs which are commonly involved. Recent genetic investigation of the disease (Gunther and Penrose, 1935; Penrose, 1936-37), however, has shown with a high degree of probability that a single dominant gene is the main causative factor. The wide variations in severity of the disorder are thought to be due to the operation of extraneous modifying factors, mainly independent genes. As is usual in cases of dominant defects which seriously impair fertility, the mutation rate of the gene is high compared with the incidence of the disease, and it seems probable that 25-50 per cent. of all cases of epiloia are directly due to a mutation in one or other parent.

Summary

1. A case of epiloia is described which, in addition to typical lesions in other organs, presented an unusual cardiac malformation in the shape of a large congenital diverticulum of the left ventricle lined by heterotopic aortic tissue.

2. This diverticulum was surrounded by heterotopic adipose tissue containing cells resembling the primitive muscle cells of a rhabdomyoma.

3. An examination of the widespread tissue abnormalities in this case justifies the conclusion that the diverse lesions of epiloia have a common dysontogenetic origin and are in no sense neoplastic.

Our best thanks are due to Dr R. J. A. Berry, director of medical services at Stoke Park Colony, for permission to record this case.

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OBSERVATIONS ON THE BACTERIOSTATIC ACTION OF SULPHANILAMIDE AND M & B 693 AND ON THE INFLUENCE THEREON OF BACTERIA AND PEPTONE

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(PLATE XV)

SOME of the experiments recorded in this article were presented to the Pathological Society of Great Britain and Ireland in January 1937 and some have been only recently executed. They illustrate in a simple fashion some of the more important points in the action of the sulphanilamide group of chemical substances and, while in part they only confirm results already published, some throw new light on the problems involved.

In these experiments only bacteriostatic power has been considered, as it appears that this is the more important aspect of sulphanilamide therapy. As regards the pyogenic cocci it seems quite unnecessary that an effective chemotherapeutic agent should be strongly bactericidal, for if it is capable of effectively restraining bacterial growth, in other words if it is actively bacteriostatic, the natural defences of the body should be capable of dealing with the infection.

Bacteriostatic power of sulphanilamide in nutrient broth

Streptococcus pyogenes "Richards." Dilutions of a culture of this organism were made in glucose broth and serial dilutions of the chemical in water. Volumes of 0.5 c.c. of a suitable dilution of culture were mixed with equal volumes of dilutions of the chemical in small test tubes and incubated at 37° C. Growth as indicated by the opacity of the culture to the naked eye is recorded in table I.

Comparison of the bacteriostatic action of sulphanilamide and M & B 693. This was done two years after the preceding experiment by the same technique. The results were essentially the same as those shown in table I.

These experiments show, as has been pointed out by a number of workers, that the bacteriostatic effect is much greater when the

implant is small and that when the implant is large the streptococci grow freely in concentrations much greater than can be obtained therapeutically.

TABLE I

Bacteriostatic power of sulphanilamide on Streptococcus pyogenes in glucose broth

Implant of cocci	Growth after 24 hours in medium containing sulphanilamide 1 in							
	200	10,000	20,000	40,000	80,000	160,000	320,000	Control: no sulphanilamide
112,000	+++	+++	+++	+++	+++	+++	+++	+++
11,200	0	0	+	++	+++	+++	+++	+++
1120	0	0	0	0	0	0	0	+
112	0	0	0	0	0	0	0	+

Staphylococci. The same technique was followed except that nutrient instead of glucose broth was used. It was found that as with streptococci there was no manifest bacteriostatic power when the inoculum was large but a great difference from the results shown in table I was that even with the smallest inoculum complete bacteriostasis was only obtained with a concentration of 1 : 12,800 sulphanilamide, which is almost the maximum obtainable in the blood therapeutically.

B. aertrycke. Here a different technique was used.

Equal parts (0.25 c.c.) of infected glucose broth and dilutions of sulphanilamide were mixed in small tubes and covered with a plug of vaseline. When *B. aertrycke* grew it formed gas from the glucose and this pushed the vaseline plug up the tube, so that some measure of the amount of growth could be obtained from the height of the gas column between the culture fluid and the vaseline plug.

The results showed that in a concentration of sulphanilamide of 1 : 40,000 there was some diminution in the amount of gas produced in 24 hours. In 1 : 6400, growth and gas production were up to 50 per cent. of that observed in the control tubes. After 96 hours, growth and gas formation occurred in the tubes containing 1 : 800 sulphanilamide. These figures show that *B. aertrycke* is very much less sensitive than *Str. pyogenes* and indicate that it is unlikely that sulphanilamide will be effective in human *B. aertrycke* infections.

Demonstration of bacteriostasis on culture plates

The bacteriostatic action of sulphanilamide can readily be shown on culture plates and also the remarkable difference which is obtained with large and small inocula of cocci.

Loopfuls of serial dilutions of the culture to be examined are streaked across a plate of a suitable medium. When these have dried into the medium

a section of the plate at right angles to the streaks is removed and the cavity so formed is filled with agar containing 1 : 300 sulphanilamide. When the plate is incubated growth appears at various distances from the sulphanilamide agar, depending on the sensitivity of the organism and the size of the inoculum.

With a sensitive microbe like *Str. pyogenes* a heavy inoculum grows right up to the sulphanilamide agar, but as the inoculum becomes smaller there is an increasing area of complete inhibition of growth, which with a very small inoculum may be as wide as 50 mm. There is, on the other hand, with an insensitive microbe such as the enterococcus, no inhibition even with the smallest inoculum.

Bacteriostatic action in serum

This was tested with staphylococcus by mixing equal volumes of infected serum and sulphanilamide or M & B 693 solution in slide cells and incubating them for 24 or 48 hours. The effect in serum was compared with the bacteriostatic effect in broth, measured as described above. The results are shown in table II.

TABLE II

Bacteriostatic action of sulphanilamide and M & B 693 on staphylococcus in serum and broth

Medium	Chemical	Growth in serum containing concentrations of chemical 1 in						Implant	Time of incubation
		2000	4000	8000	16,000	32,000	Control: no sulphanilamide		
Broth	M & B 693	0	0	+	++	+++	+++	43 cocci	24 hours
	Sulphanilamide	0	+	+++	+++	+++	+++	43 "	24 "
Serum	M & B 693	0	0	0	0	61	77	75 "	24 "
	Sulphanilamide	0	0	0	36	74	66	75 "	24 "
	M & B 693	Some minute.	63	58	67	62	83	75 "	48 "
	Sulphanilamide	79	84	81	72	73	66	75 "	48 "

+++, ++, + = degrees of opacity.

Numbers = colonies which appeared in the slide cells.

The bacteriostatic effect is more marked in serum than in broth. The effect in serum was purely bacteriostatic and not bactericidal, as in 48 hours practically the same number of colonies had grown out in the strongest solution of the chemicals (1 : 2000) as in the control containing normal salt solution. The fact that even small implants of staphylococci are only inhibited by concentrations which approach the maximum obtainable therapeutically has an important bearing on the treatment of staphylococcal infections by these drugs.

Bacteriostatic effect in de-leucocyted blood

When normal human defibrinated blood is deprived of its leucocytes by filtering through cotton wool it loses the whole of its antibacterial power towards the ordinary pyogenic cocci. Such de-leucocyted blood, therefore, offers a very suitable medium in which to test the bacteriostatic power of any chemical without any interference by the antibacterial power of the leucocytes.

I have already shown (Fleming, 1938) that in de-leucocyted blood every pneumococcus implanted grows out in the presence of M & B 693 in all concentrations up to 1 : 8000. The colonies in the stronger concentrations, however, are very minute in contrast to the large colonies which appear in the control and in the weakest concentrations. With more sensitive microbes such as *Str. pyogenes*, complete inhibition of growth takes place in concentrations which can easily be obtained in the blood therapeutically, provided the number of streptococci implanted is small. If the implant is large, growth occurs even in concentrated solutions of sulphanilamide.

TABLE III

Bacteriostatic effect of sulphanilamide on Str. pyogenes "Richards" in de-leucocyted human blood

Concentration of sulphanilamide	Number of colonies which appeared					
	Implant : 9 cocci per slide cell			Implant : 46 cocci per slide cell		
	6 hrs. at 37° C.	9 hrs. at 37° C.	+48 hrs. at room temp.	6 hrs. at 37° C.	9 hrs. at 37° C.	+48 hrs. at room temp.
Control	3	5	5	15	21	17
1 : 1,280,000	0	7	7	1	15	15
1 : 640,000	0	1	3	9	14	12
1 : 320,000	0	3	3	2	13	14
1 : 160,000	0	5	5	0	16	16
1 : 80,000	0	2	5	0	12	15
						small
1 : 40,000	0	0	0	0	1	1
1 : 20,000	0	0	0	0	0	0
	Implant 250 cocci			Implant 250,000 cocci		
	3½ hrs. at 37° C.	7 hrs. at 37° C.	24 hrs. at 37° C.	3½ hrs. at 37° C.	7 hrs. at 37° C.	
Control	53	75	75	Hundreds of minute areas of lysis in all	Complete hæmolysis in all	
1 : 12,800	0	0	12			
			small			
1 : 6,400	0	0	1			
			small			
1 : 3,200	0	0	0			
1 : 1,600	0	0	0			
1 : 800	0	0	0			
1 : 400	0	0	0			
1 : 200	0	0	0			

Table III shows the bacteriostatic effect obtained with different concentrations of sulphanilamide in human defibrinated blood infected with varying numbers of *Str. pyogenes* "Richards." Equal volumes (25 c.mm.) of the dilution of sulphanilamide and the infected blood were mixed, incubated in slide cells and examined at intervals as stated in the table.

This shows that in the first few hours there is some bacteriostasis with a concentration of sulphanilamide as low as 1:1,000,000 when the inoculum is very small. It also shows that when the implant is large the streptococci grow out in large numbers in 3½ hours, even in a concentration of 1:200.

Bacteriostatic effect in defibrinated human blood containing its full complement of leucocytes

In this medium there are two bacteriostatic agents at work, the chemical substance and the leucocytes, the latter capable of destroying considerable numbers of pyogenic cocci. Since, in the body, these chemicals act in blood containing leucocytes, examinations made in such blood are more valuable than those made in any other medium and they have the added advantage that they show whether the chemical has any deleterious action on the leucocytes. It has been found in previous experiments that most chemicals which have been used in the past as antibacterial agents in the human body have a much greater affinity for leucocytes than for bacteria and are capable of destroying leucocytic function in concentrations which have no effect on bacterial growth. This can easily be demonstrated by making a series of dilutions of the chemical and mixing equal volumes of these with suitably infected human defibrinated blood and placing the mixtures in slide cells. If a chemical like carbolic acid or quinine is used one gets three zones as the concentration of the chemical increases (Fleming, 1924, 1930-31, 1938-39): (a) an ineffective zone where the bacterial growth is exactly the same as in the control cells; (b) an antileucocytic zone where there is increased growth of the bacteria due to interference with leucocytic function by the chemical but no interference with bacterial growth; (c) an antibacterial zone where, in addition to destruction of the leucocytes, there is interference with the growth of the bacteria.

If sulphanilamide is tested in the same way with an insensitive microbe such as the enterococcus the following result is obtained.

Concentration of sulph-anilamide	1:200	1:400	1:800	1:1600	1:3200	Control
Number of colonies	188	10	0	0	0	0

The enterococcus is easily killed off by the leucocytes in human blood. In the above experiment the implant was such that it was completely destroyed by the blood without any chemical. There appeared to be no damage to leucocytic function until the concentration of sulphanilamide reached 1:400, a much higher concentration than can possibly be obtained therapeutically. With M & B 693 leucocytic function was not damaged when a

saturated solution (1 : 1000) was incubated with an equal volume of infected blood.

When, however, a sensitive microbe such as *Str. pyogenes* is used as the test organism no antileucocytic action can be seen, as

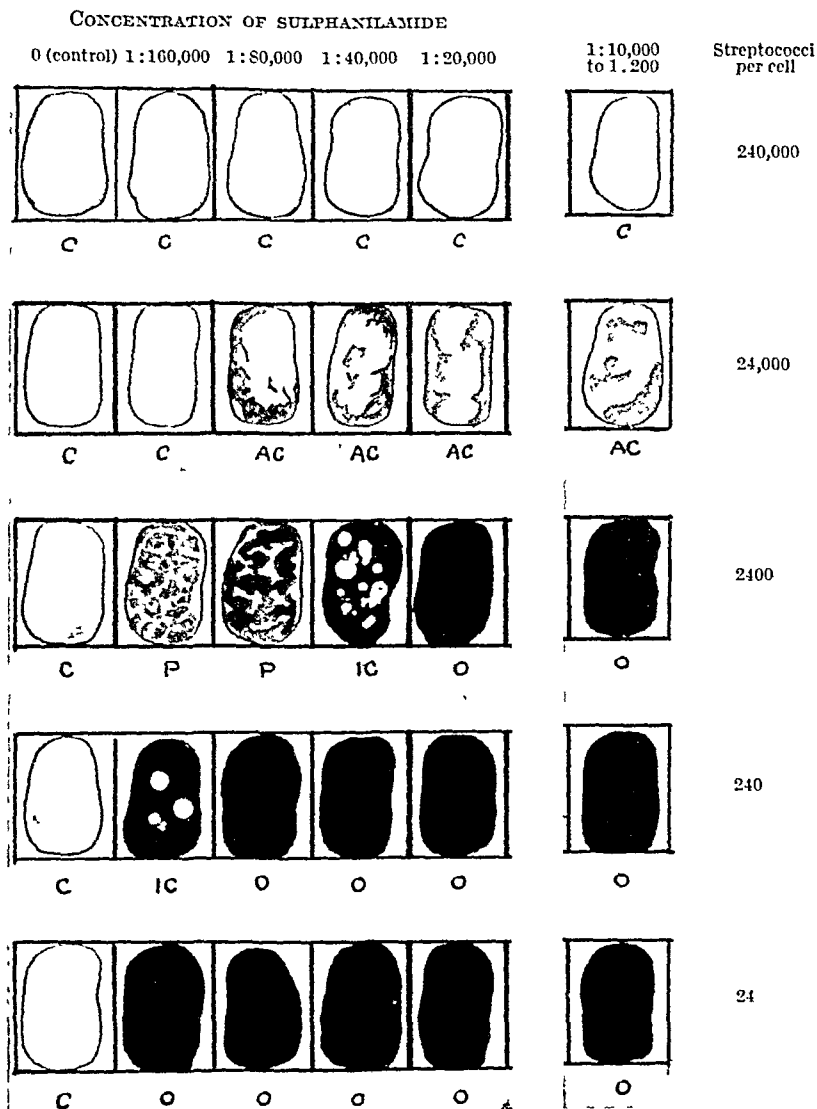


FIG. 1.—Inhibitory action of sulphaniilamide on *Streptococcus pyogenes* in human blood.

C = complete hæmolysis P = partial hæmolysis
 AC = almost complete hæmolysis IC = isolated colonies
 O = no growth

with small implants there is complete bacteriostasis with very small concentrations of the chemical. Fig. 1 illustrates the result

of a typical experiment in which 25 c.mm. of each of a series of dilutions of sulphanilamide in normal saline were incubated in slide cells with 25 c.mm. of human defibrinated blood infected with *Str. pyogenes* "Richards."

With very large implants of streptococci growth occurred in all concentrations of sulphanilamide, but as the implant decreased the bacteriostatic action of the drug became manifest, until with an inoculum of 24 cocci per cell (a number comparable with that found in the circulating blood in acute streptococcal septicæmia) there was complete inhibition in a concentration of 1:160,000, which is very much less than can be obtained therapeutically.

In another experiment with *Str. pyogenes* which I have already published (1938-39) the same degree of bacteriostasis was observed in sulphanilamide 1:400,000 and in M & B 693 1:4,000,000, showing that in human blood the latter drug was ten times more potent than the former.

Effect on the bacteriostatic power of the blood of the introduction of sulphanilamide or M & B 693 into an animal

If a drug like sulphanilamide has a direct effect on the infecting bacteria it should, when injected intravenously, *immediately* confer

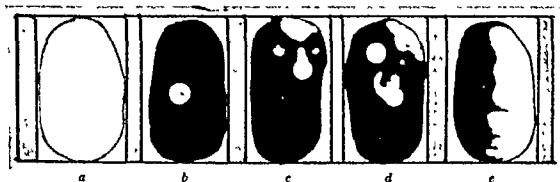


FIG. 2.—Effect of the intravenous injection of sulphanilamide on the bacteriostatic power of blood.

a, Before injection: complete hæmolysis. b, Immediately after: 1 colony. c, ½ hour after: few colonies. d, 2 hours after: few colonies. e, 5 hours after: much hæmolysis.

Same implant of streptococci in each cell.

on the blood increased antibacterial power, for it is immediately after the injection that the drug is at its maximum concentration in the blood stream. Experiments of which the following is a typical example were made to ascertain whether this did occur.

Ten c.c. of a 1 per cent. solution of sulphanilamide were injected intravenously into a rabbit. Blood was taken before and immediately after the injection and again at intervals up to 5 hours. It was defibrinated and its antistreptococcal power tested by mixing 50 c.mm. with 2.5 c.mm. of graduated dilutions of a streptococcal culture, placing the mixtures in slide cells, and noting the resulting growth. The result is shown in fig. 2.

The blood taken immediately after injection has a greatly enhanced antistreptococcal power, which has partly disappeared after 5 hours. A result of this kind indicates that the sulphanilamide acts on the bacteria without having to be changed into some other active substance in the body, for if such a change had to take place there would be an interval after the injection when the antibacterial power of the blood would be unaltered, and only after this would it be increased. For some time the mode of action of prontosil was a mystery, but a simple test of this kind would have indicated that it had to undergo some change in the body, for it is only a considerable time after its introduction into the body that the blood shows enhanced antibacterial power (Colebrook *et al.*, 1936; Hoare, 1938).

*Interference of bacteria with the action of sulphanilamide
and M & B 693*

In all the experiments on the antibacterial power of sulphanilamide or M & B 693, whether in broth, serum or blood, the concentration of the chemical necessary to inhibit growth was directly dependent on the number of bacteria in the implant. In view of this it was thought possible that the bacteria combined directly with the chemical and rendered it inert. Experiments were made by mixing large quantities of bacteria with concentrated solutions of sulphanilamide and then comparing the bacteriostatic power of the chemical thus treated with that of the original solution.

Equal parts of a 1 per cent. solution of sulphanilamide and a thick saline suspension of *Str. pyogenes* from a glucose agar plate were mixed and allowed to stand for 24 hours to allow ample time for the union of the bacteria with the chemical. The number of streptococci added was far in excess of the number which would be inhibited by that strength of the chemical. The mixture was then centrifuged to separate the streptococci and the supernatant fluid was removed and heated to kill any remaining living streptococci. At the same time a second volume of 1 per cent. sulphanilamide was diluted with an equal volume of normal salt solution, incubated for 24 hours and then centrifuged and boiled as a control along with the sulphanilamide-streptococcus tube.

The bacteriostatic power of these two fluids was tested on *Str. pyogenes* by mixing equal parts of the fluid with human defibrinated blood suitably infected with the "Richards" streptococcus and incubating the mixtures in slide cells.

It was found that with the sulphanilamide-streptococcus supernatant there was copious growth of the streptococci, whereas there was complete inhibition with the sulphanilamide-saline. It was clear therefore that the streptococci had in some way prevented the bacteriostatic action of the sulphanilamide.

In the next experiment serial dilutions of the two fluids were made, each of which was tested in exactly the same way as the

BACTERIOSTATIC ACTION OF SULPHANILAMIDE

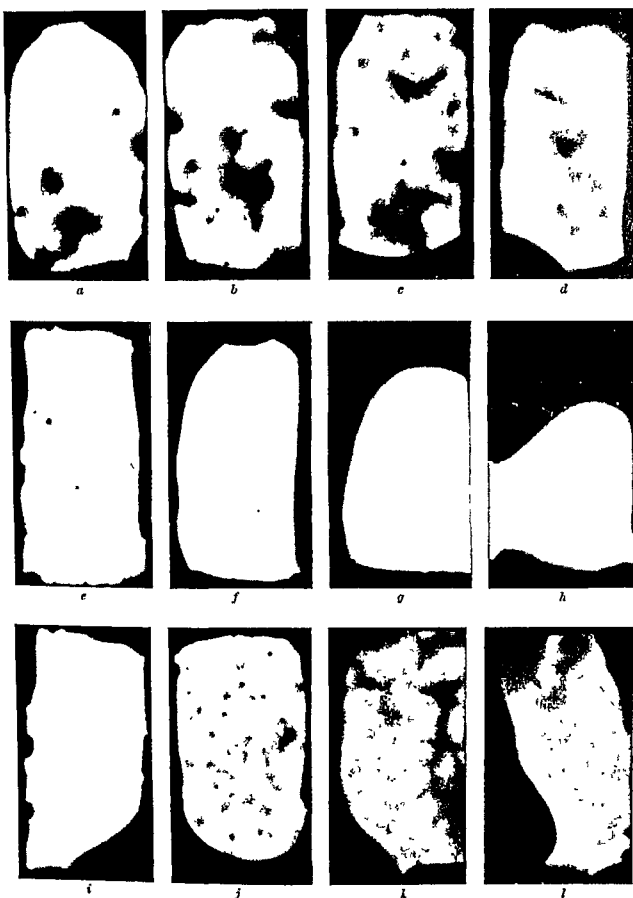


FIG. 3.—Effect of a thick suspension of streptococci on the bacteriostatic power of sulphanilamide. The figures give the final concentration of sulphanilamide in the slide cells. The shaded areas represent hæmolysis around streptococcus colonies.

a.	0 (control).	Areas of hæmolysis
b.	1:400,000.	Areas of hæmolysis
c.	1:200,000.	Areas of hæmolysis
d.	1:100,000.	Few hæmolytic areas
e.	1:50,000.	Two colonies
f.	1:25,000.	No growth

g.	1:12,800.	No growth
h.	1:6400.	No growth
i.	1:3200.	No growth
j.	1:1600.	Many colonies
k.	1:800.	Many areas of hæmolysis
l.	1:400.	Many hæmolytic areas

undiluted fluids in the previous experiment. Again it was found that the undiluted supernatant had lost its bacteriostatic power, but the interesting point emerged that as this was diluted in saline the bacteriostatic power gradually returned. We were thus faced with the anomalous situation that the supernatant containing 1:200 sulphanilamide had no bacteriostatic power, whereas when this was diluted until the concentration was reduced to about 1:10,000 there was complete inhibition of the streptococci. The ultimate dilution at which bacteriostasis was manifest with the sulphanilamide-streptococcus supernatant was almost the same as it was with the sulphanilamide-saline control. It was obvious that the sulphanilamide was not bound to the streptococci, otherwise on centrifuging the mixture all or most of the chemical would have been removed, whereas in this experiment little or none disappeared. The appearance of the growth obtained in such an experiment is shown in fig. 3.

Experiments were then made to see whether dead organisms inhibited the bacteriostatic action in the same way as living. A thick suspension of streptococci was divided into two parts, one of which was boiled for 5 minutes. Each was then mixed with sulphanilamide 1 per cent. and treated and tested as above described. The results obtained with the living and dead cocci were exactly the same.

The effect of other organisms on sulphanilamide

Sulphanilamide was mixed with thick suspensions of various organisms and the mixtures were treated and tested as in the previous experiments. The results obtained are given in table IV.

TABLE IV

Inhibition of bacteriostatic action of sulphanilamide

Sulphanilamide treated with	Number of colonies growing in the blood-supernatant mixtures containing an ultimate concentration of sulphanilamide of 1 in							
	400	800	1600	3200	6400	12,800	25,600	Control
<i>B. aertrycke</i> .	60	63	62	55*	0	0	0	64
<i>Str. viridans</i> .	71	71	46	37*	0	0	0	58
<i>Str. pyogenes</i> .	67	71	71	30*	0	0	0	58
<i>Staphylococcus</i>	67	52	53	30*	0	0	0	56
Sterile broth .	52	0	0	0	0	0	0	62
Normal saline .	0	0	0	0	0	0	0	55

* Small colonies with little hæmolysis.

They show that a variety of bacteria have exactly the same effect on the bacteriostatic power of sulphanilamide and that sterile nutrient broth had a similar but less powerful action. The results

were of the same order whether the blood used for the final test contained leucocytes or not, so that they could not be due to any action of the bacteria on the leucocytes. There was no other conclusion possible than that the bacteria acted on the sulphanilamide.

These findings were presented to the Pathological Society of Great Britain and Ireland at their meeting in January 1937, but were not otherwise published as there seemed at the moment no satisfactory explanation of the facts observed. The experiments have recently been repeated with M & B 693 with exactly similar results.

In 1938 Lockwood described the influence of peptone on the bacteriostatic action of sulphanilamide. As his findings appeared somewhat similar to mine with bacteria and sulphanilamide, it was determined to test the effect of peptone solution by the technique I had previously used.

Peptone (1.25 per cent.) was mixed with an equal volume of sulphanilamide (1 per cent.) and serial dilutions of the mixture were added to human defibrinated blood suitably infected with the "Richards" streptococcus and incubated in slide cells.

The undiluted peptone-sulphanilamide mixture had no bacteriostatic power, but when it was diluted sixteenfold with normal saline this became manifest and was maintained until the concentration of sulphanilamide was reduced to 1:200,000, which is about the limit of bacteriostasis of a simple solution of sulphanilamide in blood. The result, therefore, is precisely similar to that previously obtained with thick suspensions of bacteria (*cf.* fig. 3).

Another similar experiment was done in which sulphanilamide (1 per cent.) was treated with different strengths of peptone and with a thick suspension of staphylococcus. The results are given in table V.

There is no essential difference in the results obtained with peptone and with bacteria. The stronger the peptone the greater is the inhibition of the bacteriostatic action of sulphanilamide, and the antibacteriostatic effect disappears when the concentration of peptone is reduced to 1:5000 irrespective of the concentration of sulphanilamide. For the sake of clarity the concentrations of peptone and sulphanilamide in which the antibacteriostatic action disappeared in each series are given in table VI.

Lockwood has put forward the hypothesis that sulphanilamide acts on streptococci by inhibiting the ferments by which they break down the proteins of blood fluids into essential foodstuffs and that when peptone is added the streptococci can use this predigested material and grow out even when sulphanilamide is present. He suggests also that the reason for the remarkable difference between the bacteriostatic power of these chemicals on large and small inocula is that some of the streptococci of a large inoculum are destroyed and in their dissolution liberate a peptone-like substance which acts in a similar way to added peptone and allows the remaining streptococci to grow.

TABLE V
Effect of different concentrations of peptone on the bacteriostatic power of sulphani-
lamide

Growth of <i>Str. pyogenes</i> "Richards" in equal parts of human blood and dilution of sulphani- lamide-peptone or sulphani- lamide-staphylococcus mixture									
Dilution of sulphani- lamide- peptone mixture	Final con- centration of sulphani- lamide	Control (no peptone)	Original concentration of peptone 1.25 per cent.		Original concentration of peptone 2.5 per cent.		Original concentration of peptone 5 per cent.		Sulphani- lamide treated with staphylococcus
			Growth	Final concentration of peptone	Growth	Final concentration of peptone	Growth	Final concentration of peptone	
Control	0	+++	+++	0	+++	0	+++	0	++
1:1024	1:800,000	+	+	1:160,000	++	1:80,000	++	1:40,000	++
1:512	1:400,000	±	±	1:80,000	+	1:40,000	+	1:20,000	+
1:256	1:200,000	0	0	1:40,000	0	1:20,000	±	1:10,000	+
1:128	1:100,000	0	0	1:20,000	0	1:10,000	0	1:5000	0
1:64	1:50,000	0	0	1:10,000	0	1:5000	+	1:2560	0
1:32	1:25,000	0	0	1:5000	0	1:2560	±	1:1280	++
1:16	1:12,800	0	0	1:5000	+	1:1280	+++	1:640	++
1:8	1:6400	0	±	1:2560	+++	1:640	+++	1:320	++
1:4	1:3200	0	+++	1:1280	+++	1:320	+++	1:160	++
1:2	1:1600	0	+++	1:640	+++	1:160	+++	1:80	++
1:1	1:800	0	+++	1:320	+++	1:80	+++	1:40	++

+++ = several hundred colonies.

± = few small colonies.

TABLE VI

Antibacteriostatic action of peptone on sulphanilamide.

Undiluted sulphanilamide-peptone-blood mixture		Lowest dilution showing no anti-bacteriostatic effect	Concentration in this dilution of	
Concentration of			sulphanilamide	peptone
sulphanilamide	peptone			
1 : 400	1 : 320	1 : 16	1 : 6400	1 : 5000
1 : 400	1 : 160	1 : 32	1 : 12,800	1 : 5000
1 : 400	1 : 80	1 : 64	1 : 25,000	1 : 5000
1 : 400	1 : 40	1 : 128	1 : 50,000	1 : 5000

These results are in agreement with Lockwood's findings.

It is clear from the experiments detailed above that dead streptococci and other organisms have the same apparent effect as peptone on the bacteriostatic action of sulphanilamide or M & B 693. Quantitative experiments have shown, however, that it requires a much larger number of dead streptococci to inhibit the bacteriostatic action of a concentrated solution of sulphanilamide on a small inoculum of living *Str. pyogenes* in human blood than the number of living streptococci which will grow out if implanted into the same human blood containing the same concentration of sulphanilamide.

A direct experiment was made to see whether dead streptococci added to human blood aided the growth of a small inoculum of living *Str. pyogenes* in the absence of sulphanilamide.

A thick suspension of streptococci was made in normal saline and boiled for five minutes. Serial dilutions of this suspension (1 : 2, 1 : 10, and 1 : 100) were made and to 25 c.mm. of each were added 25 c.mm. of de-leucocyted human blood infected with 147 streptococci of the strain used to make the killed suspension. A control mixture was also put up containing normal saline instead of the streptococcal suspension. These mixtures were incubated in slide cells.

It was found that at the end of five hours growth and hæmolysis of the blood were visible only in the cells which contained the streptococcal suspensions and were most vigorous in the cell containing the undiluted suspension. There was no growth nor hæmolysis in the control cell. This shows that dead streptococci do liberate into blood some substance or substances which favour the growth of the homologous streptococcus.

Since these experiments were made a very interesting paper has been published by Stamp (1939), who has shown that a fraction can be isolated from hæmolytic streptococci which has a very powerful inhibitory effect on the bacteriostatic action of sulphanilamide or M & B 693. He found that this purified fraction even when diluted 1 : 1,000,000 caused an appreciable acceleration of the growth of *Str. pyogenes* in blood. There is nothing in Stamp's findings which is in disagreement with the results I have obtained but he has carried the subject further in regard to the hæmolytic streptococcus by isolating the active fraction.

Summary

Experiments are described showing the bacteriostatic power of sulphanilamide and M & B 693 in broth, serum and blood, with or without leucocytes, and on solid culture medium.

All such experiments show that these substances have a powerful bacteriostatic action on small numbers of *Str. pyogenes* but that the action is negligible on massive implants.

Sulphanilamide injected intravenously into a rabbit immediately confers on the blood an increased bacteriostatic power. The significance of this is discussed.

Thick suspensions of bacteria in combination with sulphanilamide or M & B 693 give off substances which inhibit the bacteriostatic action of the chemical but on dilution the bacteriostatic power returns.

Peptone added to sulphanilamide has a similar effect and the degree of inhibition of the bacteriostasis appears to depend on the concentration of the peptone and to be independent (within limits) of the concentration of sulphanilamide.

Dead streptococci added to human blood favour the growth of small implants of the same streptococci.

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COAGULASE PRODUCTION AS A CRITERION FOR
THE CLASSIFICATION OF THE STAPHYLOCOCCI

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THE classification of the genus *Staphylococcus* is at present primarily based on the production of pigment but this method is generally considered to be unsatisfactory. The main objection is that pigment production is not constant but is dependent on a number of variable factors such as temperature of incubation, atmospheric conditions and the nature of the medium. Difficulty in determining the type of pigment is consequently not uncommon. Other methods have been tried, using biochemical, serological, hæmolytic and pathogenic activities as a basis, but these have proved of limited value for general application.

In recent years much evidence has been obtained that pathogenic strains have the apparently unique property of producing a substance, staphylocoagulase, which is able to coagulate plasma. von Daranyi (1926) observed that this power was possessed by *albus* as well as *aureus* strains and was an important criterion of pathogenicity. Chapman *et al* (1934), after an investigation of more than 2500 strains, concluded that staphylococci capable of coagulating plasma were probably pathogenic, irrespective of the type of pigment produced. Cruickshank (1937) found that staphylocoagulase was produced only by pathogenic strains, whether *aureus* or *albus*, and recommended coagulase production as a test for pathogenicity. Human plasma was more satisfactory than that from animal sources. Cowan (1938), investigating a number of strains from human and animal sources, found that there was an absolute correlation between the production of hæmolysin (α and β) and coagulase, the formation of hæmolysins being an important criterion of pathogenic strains. Flaum (1938) also observed that the power of coagulating plasma was possessed only by pathogenic strains.

The main characters of pathogenic strains of *Staphylococcus* are generally accepted as being the production of soluble hæmolysins, pathogenicity for rabbits and elaboration of staphylocoagulase. Tests to demonstrate hæmolysin production and pathogenicity for rabbits involve much time and expense and are not suitable for general application in routine work. The demonstration of coagulase on the other hand is simple and the test can be applied in a routine laboratory without much difficulty. It is, however,

not mentioned by Bergey *et al.* (1939). I have investigated the suitability of the test for the differentiation of pathogenic and non-pathogenic strains of staphylococci in routine work.

Technique

There is no standard method for carrying out the coagulase test; citrated human plasma undiluted or diluted from 1:2 to 1:10 with saline or broth has been used by different workers. After various trials, a fixed dilution of one part of citrated plasma (0.5-1.5 per cent. citrate) and two parts of physiological saline was used in this work. This was inoculated with either a loopful of a 24-hour growth on agar or blood agar or with several drops of a 24-hour culture in glucose broth and incubated at 37° C., readings being made at hourly intervals for 4-6 hours and finally after 18-24 hours.

RESULTS

Factors concerned in the coagulase test

The nature of staphylocoagulase has not been definitely determined but it is generally accepted as being an enzyme. Attempts to demonstrate antigenic properties have failed. I have found that plasma from patients with high antihæmolysin and antileucocidin titres was invariably coagulated, even undiluted, by active strains.

Cruikshank observed that plasma kept for 10 days was quite satisfactory. A sample was kept at 0°-2° C. and examined weekly: after 6 weeks coagulation was still obtained. Thus suitable plasma can readily be conserved at low temperatures.

A convenient source of human plasma is blood sent to the laboratory for urea estimation. Samples were therefore collected from blood showing a wide range of urea values; in no instance did urea in concentrations of 26-340 mg. per 100 c.c. interfere with the coagulation.

Another factor influencing the reaction is the age of the culture. Cultures were left at room temperature for varying periods of time and then examined. Marked variation was found with different strains; in some cases plasma was slowly coagulated by strains 120 days old, in others the power was temporarily lost after standing for only 20-30 days but was regained on subculture. The test should therefore be carried out with fresh young cultures, as these multiply more readily and so are able to give a greater and more rapid production of coagulase.

A satisfactory method of carrying out the test in a routine laboratory is consequently to prepare 1:3 dilutions of plasma collected from blood urea specimens and to inoculate heavily with a young culture of the organisms to be examined. Some variation may be found in different batches of plasma and therefore positive and negative controls should always be made. The degree of coagulation may also vary: in some cases the diluted plasma

becomes solid, while in others there is only a fine but definite coagulum, either floating in the fluid or settling as a deposit. As coagulum formation may occasionally occur slowly it is advisable to continue incubation overnight.

Relationship of coagulase production to other biological activities of the staphylococcus

Staphylococci isolated from lesions, from apparently normal tissues and from air contaminated culture media have been examined for coagulase production and in many cases for pigment production, fermentation of mannitol, the production of hæmolysis on blood agar plates and also by slide agglutination.

(a) *Staphylococci isolated from lesions.* Strains have been isolated from a wide variety of lesions, mainly boils, carbuncles and acute osteomyelitis, and from the blood in generalised infections. The results obtained with these have been arranged in table I.

TABLE I

✓ *Reactions of staphylococci from active infections*

Pigment	Coagulase production		Mannitol fermentation		Serological types				
	Tested	Positive	Tested	Positive	1	2	3	Atypical	0
<i>Aureus</i>	120	120	66	64	18	10	26	8	2
<i>Albus</i>	7	4	7	5	1	0	3	0	2
<i>Citreus</i>	0		0		0	0	0	0	0

Coagulase production. 120 *aureus* strains from active lesions all produced coagulase, as did 4 out of 7 *albus* strains. Of the 3 *albus* strains giving negative results one was isolated along with *C. acnes* from a case of acne and was probably a secondary participant in the infection, another was obtained in pure culture from a minute superficial pustule and the third was grown in pure culture from urine collected from a case of doubtful cystitis. Thus these strains were of either low or doubtful pathogenicity and were probably saprophytes growing at the expense of dead or damaged tissue.

Hæmolysis on blood agar plates. Irregular results were obtained by this method. Several *aureus* strains did not show zones of hæmolysis in 24 hours, while saprophytic *albus* strains not infrequently gave well marked hæmolysis. This method was consequently abandoned.

Mannitol fermentation. It is well recognised that mannitol is generally fermented by pathogenic strains with the production of acid but no gas; the results in table I confirm this observation. Moreover the fermentation was almost invariably present by the third day; in contrast, when mannitol is fermented by saprophytic strains, this frequently occurs only after 5 days.

Pigment production. Staphylococci have long been differentiated by pigment production into three main types, *aureus*, *albus* and *citreus*, *aureus* strains being highly pathogenic, *albus* moderately or slightly so, and *citreus* non-pathogenic. This method of classification is unsatisfactory for many reasons. It is often difficult in young cultures to determine the nature of the pigment, incubation at 37° C. for 24 hours followed by room temperature for at least 24-48 hours being frequently necessary for the development of the pigment. Also there is no distinction between pathogenic and non-pathogenic strains of *Staph. albus*, while some highly pigmented *aureus* strains may not be pathogenic. Moreover *albus* variants may be obtained from *aureus* strains and still give the other reactions of the parent strain.

Serology. Efforts to subdivide staphylococci by ordinary agglutination and precipitation methods have not been entirely satisfactory, but Cowan (1939), using a slide agglutination technique, has found that pathogenic staphylococci can be divided into three main types. A number of strains gave atypical results but agglutination never occurred with non-pathogenic strains.

Tests were carried out with Cowan's three type sera by his technique, the essential points of which are the use of a 24-hour culture in 0.05 per cent. glucose broth, heated at 100° C. for a few minutes, cooled and centrifuged, the deposit being resuspended in a small amount of broth. A loopful of serum is mixed on a slide with a drop of suspension and readings are made within a few minutes with the hand lens.

The test proved easy to carry out and little difficulty was experienced in making readings. Positive results were given only by coagulase-positive strains, irrespective of the type of pigment produced. Type II strains were easily distinguished but the differentiation of type I from type III was often extremely difficult. There must be considerable overlapping in the antigenic structure of these two types. Moreover a batch of type III serum which had become somewhat inspissated gave marked agglutination with type I strains. It is thus obvious that while slide agglutination has distinct possibilities, further work must be carried out before agglutination of staphylococci can occupy the same status as it does in the typing of pneumococci.

(b) *Staphylococci from other sources.* Strains in this group were obtained from the nasal and pharyngeal mucosa or the urine of apparently healthy individuals and from air-con-

taminated culture media. In no instance was there any indication that the organisms were concerned with a pathological process. The results are given in table II.

TABLE II

✓ Reactions of strains present as commensals or contaminants

Pigment	Coagulase production		Mannitol fermentation		Serological types				
	Tested	Positive	Tested	Positive	1	2	3	Atypical	0
<i>Aureus</i> . . .	27	22	21	17	2	2	7	0	2
<i>Albus</i> . . .	80	3	44	13	1	0	0	0	19
<i>Citrus</i> . . .	15	0	14	0	0	0	0	0	0
Other organisms .	11	0	0	0	0	0	0

Coagulase production. Except on three occasions coagulase was produced only by *aureus* strains, which were invariably isolated from either urine or throat and nasal swabs and were undoubtedly potential pathogens. The common habitat of the staphylococcus, especially of pathogenic strains, is the naso-pharyngeal mucosa, particularly in cases with chronic staphylococcal infections. Many of the *aureus* strains were actually isolated from patients suffering from some chronic staphylococcal infection such as furunculosis. *Aureus* strains which gave negative results were highly pigmented and were invariably isolated from normal urine.

Albus or *citrus* strains, which were usually air contaminants, gave mainly negative results. Three *albus* strains isolated from throat swabs gave a positive result. One was agglutinated with type I serum. Type sera did not agglutinate any of the other strains examined.

Several other species of bacteria, including *Streptococcus hæmolyticus*, *Streptococcus faecalis*, *M. tetragenus*, *Bact. paratyphosum B*, *Proteus*, *N. pharyngis*, *N. catarrhalis*, *Sarcina*, diphtheroid bacilli and *Bact. alkalescens*, were also examined for coagulase production, with negative results.

DISCUSSION

These results confirm the observation that coagulase is formed only by pathogenic strains of the staphylococcus. Coagulase production therefore constitutes an important criterion for classification and it is suggested that the genus should be primarily subdivided on this basis into coagulase-positive and coagulase-negative strains. *Coagulase-positive* strains are pathogenic, usually ferment mannitol, may produce *aureus* or *albus* pigment, form soluble hæmolysins and should be designated *Staphylococcus pyogenes*.

Coagulase-negative strains are non-pathogenic, usually non-mannitol fermenters, tend to form *albus* and *citreus* pigments, do not produce soluble hæmolysins and should be designated *Staphylococcus saprophyticus*.

Subdivision of *Staph. saprophyticus* can be carried out on the basis of pigment production and of *Staph. pyogenes* by pigment production or serology.

The power to produce soluble hæmolysins is an important criterion for differentiating these two groups but does not lend itself to general application. There are several hæmolysins requiring special cultural requirements for their production. Moreover it has not infrequently been considered that hæmolysis on a blood-agar plate is an index of the formation of soluble hæmolysins, as with the streptococci. Satisfactory evidence of this has never been produced and most workers have found that non-pathogenic strains of staphylococci may cause hæmolysis on blood agar plates, while pathogenic strains may not do so. The use of such terms as "hæmolytic *Staphylococcus aureus*," based on information obtained by blood agar plate, is therefore to be condemned.

SUMMARY

The use of the coagulase test for the classification of staphylococci is discussed and is recommended for general application.

It is suggested that coagulase-positive strains should be designated *Staphylococcus pyogenes* and coagulase-negative strains *Staphylococcus saprophyticus*.

I wish to thank Dr S. T. Cowan for supplying the type sera used in the agglutination tests.

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THE EFFECT OF THE DESENSITISATION OF TUBERCULOUS GUINEA-PIGS

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THE experiments here described were undertaken in an endeavour to examine the truth of the thesis propounded by Rich and his colleagues (Rich, 1929, 1930, 1931, 1933 *a* and *b*; Rich and McCordock, 1929; Rich and Brown, 1929-30; Rich, Chesney and Turner, 1933; Rich, Jennings and Downing, 1933) that allergy plays no part in immunity. We need do no more now than state that the conclusions we have reached from the results of our own experiments on tuberculosis during the past five years and from a review of the literature are not in accordance with this view.

SENSITISATION OF GUINEA-PIGS WITH KILLED TUBERCLE BACILLI

Virulent strains of human or bovine tubercle bacilli were cultured on egg medium for about a fortnight. The organisms were removed, ground up in glass-distilled water, standardised by opacity (Wilson and Schwabacher, 1936-37) and killed by heat. Thin suspensions were exposed in a water-bath to a temperature of 60°-70° C. for 1½ hours; thicker suspensions were placed in the steamer for 1 hour on two successive days; very thick suspensions were autoclaved at 1½ atmospheres for 25 minutes. Control experiments showed that these exposures could be relied upon to destroy all living organisms.

Twenty-six guinea-pigs were inoculated intramuscularly into the thigh with varying doses of these killed suspensions at 5-7 day intervals for 12-20 weeks. Skin sensitivity to 0.2 ml. of Old Tuberculin (O.T.) was tested intradermally at frequent intervals. The reactions were read after 24 hours. No reaction was regarded as positive unless either (1) the diameter of the combined area of redness and induration was 20 mm. or over or (2) there was an area of redness and induration of 15 mm. diameter accompanied by central blanching.

Every animal became allergic, but some died before a high degree of allergy had been attained. The time taken to become sensitive to 1:10 tuberculin varied from 3 to 8 weeks and the total dosage received from 2.5 to 60 mg. With a test dose of 1:1000 tuberculin the time was 11-22 weeks, and the total dosage 1.5-88 mg. With 1:10,000 tuberculin the time was 17-26 weeks and the total dosage 10.5-100 mg. Animals dying during the period of observation were carefully examined. Beyond a local

caseous lesion, which so frequently follows the injection of large numbers of dead bacilli, no abnormality was found.

These experiments merely confirmed those of previous workers in showing that guinea-pigs can be readily sensitised to tuberculin by dead tubercle bacilli. It was difficult to draw any definite conclusions as to the relative importance of dosage and time, but it seemed clear that fairly high degrees of sensitivity could be reached by inoculation of relatively small numbers of bacilli. Confirmation of this conclusion will be provided in the next two sections.

*Loss of sensitivity in sensitised guinea-pigs with
the passage of time*

Six guinea-pigs were inoculated with 1·5, 5, 10, 20, 30 and 60 mg. of killed tubercle bacilli respectively. The first three animals received their total dosage in a single inoculation; the last three received theirs in two inoculations at an interval of nine days. The record of tuberculin sensitivity, judging the time from the first dose, was as follows.

After 6 weeks	6 out of 6 animals reacted to 1 : 10 tuberculin.
„ 8 „ 5 „ 6 „ „ 1 : 100 „	
„ 11 „ 6 „ 6 „ „ 1 : 1000 „	
„ 6 months 2 „ 5 „ „ 1 : 10,000 „	
„ 8 „ 5 „ 5 „ „ 1 : 1000 „	
„ 9 „ 5 „ 5 „ „ 1 : 100 „	
„ 10 „ 3 „ 4 „ „ 1 : 10 „	
„ 11 „ 1 „ 2 „ „ 1 : 10 „	

One animal died after 15 weeks and 3 after 9-11 months; 2 were killed at the end of the experiment. With the exception of the first animal, which had a local caseous lesion, none showed any abnormality on post-mortem examination. The regional lymphatic glands were not enlarged nor could any acid-fast bacilli be found on microscopical examination of the viscera.

The experiment was on a very small scale but the results were quite clear. Animals sensitised with killed tubercle bacilli gradually lose their sensitivity with the passage of time.

*Desensitisation of sensitised guinea-pigs by
artificial means*

Four guinea-pigs were inoculated twice at an interval of six weeks with 1 mg. of heat-killed tubercle bacilli. Eight weeks after the first inoculation they all reacted to 1 : 100 tuberculin. They were then given nine subcutaneous injections of O.T. in a fortnight, equivalent in all to 0·85 c.c. of the undiluted product. Two days after the last inoculation they were all negative to 1 : 10

tuberculin The animals were left without further treatment A fortnight later they were positive to 1 · 10, and six weeks later to 1 100 tuberculin

Three guinea-pigs were given a single dose of 2 mg of heat-killed tubercle bacilli Eight weeks later they all reacted to 1 1000 tuberculin They were then given twelve subcutaneous injections of O T in a fortnight, equivalent in all to 1·1 c.c of the undiluted product At the end of this course none of the animals reacted to 1 10 tuberculin They were left without further treatment Nine days and again 4 weeks later they reacted to 1 10 tuberculin and after another 4 weeks to 1 100 tuberculin

In neither experiment did any of the animals show any evidence of tuberculosis *post mortem*

Effect of sensitisation and desensitisation on experimental tuberculosis in guinea pigs

The aim of the four experiments now to be described was to find out the effect of large doses of tuberculin, given either before or after infection, on the progress of experimental tuberculosis in sensitised and control guinea pigs

The guinea pigs required for each experiment were divided as evenly as possible according to sex, weight and colour into the requisite number of groups A few extra animals were usually included in case of deaths before infection The weights varied to some extent in the different experiments, in expts 1 and 2 the average at the time of infection was about 400 g, in expt 3 about 320 g and in expt 4 about 650 g Sensitisation was accomplished by the intramuscular inoculation into the left thigh of one or more doses of heat killed tubercle bacilli Desensitisation was carried out by giving increasing doses of O T up to a given maximum, and repeating this maximum dose five or six times a week The O T used in the first three experiments was prepared by ourselves from strain T 100, kindly supplied by the late Captain S R Douglas and was standardised against tuberculin of known potency In the fourth experiment the O T used was prepared by the Wellcome Physiological Research Laboratories

All animals were infected by intramuscular inoculation into the right thigh with a dose of 0 000001 mg of a virulent human strain grown for about a fortnight on egg medium, a viable count was made on the inoculum After infection the animals were observed till death Graded intradermal tuberculin tests were made as a rule once a fortnight, and except in expt 4 the inguinal glands on the side of the infection were palpated at frequent intervals *Post mortem* a careful examination was made of the extent and severity of the lesions and quantitative lung cultures were put up as described by Schwabacher and Wilson (1936 37)

Experiment 1

15 11 34 Groups A and B were sensitised with human tubercle bacilli killed by heating at 66° C for 90 minutes Two doses, 2 5 and 7 5 mg, were given at 19 days' interval Five weeks after the second dose most of the animals reacted positively to an intradermal inoculation of 1 1000 tuberculin Group A received no further treatment

TABLE I

Desensitisation and infection in experimental tuberculosis of the guinea-pig (expt. 1)

Group	Total dosage of killed tubercle bacilli		No. of animals infected	Average dosage of O.T. (c.c.)		Tuberculin sensitivity		Survival time in days		Extent and severity of lesions post mortem					Colonies of T.B. in lung cultures	
	Amount	No. of doses		Before infection	After infection	Before infection	After infection	A.M.	S.D.	+++	++	+	0	Minimum	Maximum	G.M.
A	10 mg.	2	9	1 : 100,000	Fell to 1 : 10,000 or 1 : 1000	107.4	57.8	7	2	0	0	1.2 × 10 ⁶	460 × 10 ⁶	38 × 10 ⁶
B	10 mg.	2	9	1.8	32.8	Weak 1 : 10	...	147.9	62.2	0	1	7	1	0	132 × 10 ⁶	510,000
C	8	Rose to 1 : 10,000 or 1 : 100,000	129.1	42.8	8	0	0	0	120,000	210 × 10 ⁶	10.6 × 10 ⁶

A.M. = arithmetic mean S.D. = standard deviation G.M. = geometric mean (tables I-IV).

	Between groups		
	A and B	A and C	B and C
Observed difference in mean survival time in days .	40.5	21.7	18.8
Standard error of difference	30.0	26.6	27.3
Observed difference divided by its standard error .	1.4	0.8	0.7

7.1.35. Group B was given 5 or 6 inoculations of O.T. per week, the dose rising from 0.005 to 0.3 c.c. After 4 weeks the animals in group B were almost completely desensitised, giving only a weak reaction to 1:10 tuberculin. On the other hand the untreated animals in group A reacted to 1:100,000 tuberculin.

2.2.35. Along with a group of controls, all animals were infected with a dose of approximately 12 viable tubercle bacilli.

After infection groups A and C were left untreated. The animals in group B received injections of O.T. five times a week till death. For 3½ months they were given 0.3-0.4 c.c. per day; thereafter the dose was reduced to 0.2 c.c., as this was found sufficient to keep them continuously desensitised to 1:10 tuberculin.

The main results of this experiment are recorded in table I.

In group B the glands became enlarged very much later than in groups A and C. For example, 6 weeks after infection 7 out of 9 animals in group A, 6 out of 8 in group C, and only 1 out of 8 in group B had palpably enlarged inguinal glands.

In group A the tuberculin sensitivity fell almost immediately after infection from 1:100,000 to 1:10,000; later in some animals it fell to 1:1000. In group C sensitivity to 1:10 tuberculin was established 4 weeks after infection; it rose to a maximum of 1:10,000 or 1:100,000 12 weeks after infection and remained more or less constant at this level till death.

The average survival time was greatest in group B, but the differences between the three groups were never significant. In groups A and C lesions described as "severe" or "massive" were present at autopsy in every animal, and generally affected the spleen, liver and lungs; in group B, only one animal had lesions described as severe and these were restricted to the lungs. Except for two animals which showed a late military spread, the lesions in group B were on the whole so slight that it is difficult to believe that the animals died of tuberculosis.

The mean number of colonies of tubercle bacilli cultivated from the lungs was much smaller in group B than in the other two groups.

The results of this experiment strongly suggest that the effect of continuous desensitisation on the animals in group B was to limit very considerably the progress and spread of the disease.

Experiment 2

14.2.35. Groups A and B were sensitised with human tubercle bacilli killed by heating at 100° C. for 1 hour on two successive days or by autoclaving at 1½ atmospheres for 25 minutes. Six doses were given over a period of 3 months, the total dosage amounting to 15.5 mg. At the conclusion of this course the animals reacted to the intradermal injection of 1:100 tuberculin.

13.5.35. Group A was left untreated. Group B received 16 doses of O.T. within 3 weeks, rising from 0.01 to 0.2 c.c. At the end of this time all

TABLE II
Desensitisation and infection in experimental tuberculosis of the guinea-pig (expt. 2)

Group	Total dosage of killed tubercle bacilli		No. of animals infected	Average dosage of O.T. (c.c.)		Tuberculin sensitivity		Survival time in days		Extent and severity of lesions post mortem					Colonies of T.B. in lung cultures		
	Amount	No. of doses		Before infection	After infection	Before infection	After infection	A.M.	S.D.	+++	++	+	0	Minimum	Maximum	G.M.	
A	15.5 mg.	6	10	1 : 100	Rose to 1 : 10,000 or 1 : 100,000	132.9	74.1	4	3	3	0	0	2700×10^6	5.6×10^6	
B	15.5 mg.	6	8	1.9	24.6	169.8	95.7	0	4	4	0	10,500	2600×10^6	17.8×10^6	
C	10	1.8	23.4	158.8	72.9	3	3	4	0	22,000	$140,000 \times 10^6$	18.6×10^6	
D	6	Rose to 1 : 1000. 1 : 100,000	86.5	29.2	3	1	2	0	71,000	3200×10^6	16×10^6	

	Between groups					
	A and B	A and C	A and D	B and C	B and D	C and D
Observed difference in mean survival time in days	36.9	25.9	46.4	11.0	83.3	72.3
Standard error of difference	43.8	34.7	27.9	43.6	38.4	27.6
Observed difference divided by its standard error	0.84	0.75	1.7	0.25	2.2	2.6

animals but one were completely desensitised to 1:10 tuberculin, while in group A 9 animals reacted to 1:100 and one to 1:1000. Group C, of normal animals, received in the same time 2 doses of 0.1 c.c. and 8 of 0.2 c.c. of O.T.

4.6.35. The animals were infected, along with group D (controls), with approximately 40 living organisms.

After infection the animals in groups B and C were given 5 subcutaneous injections of 0.2 c.c. of undiluted O.T. per week. This treatment succeeded in keeping the animals in group B continuously desensitised to 1:10 tuberculin and in preventing those in group C from becoming sensitised.

The main results of this experiment are recorded in table II.

The rate of enlargement of the inguinal glands was greatest in group D and least in groups B and C; group A occupied an intermediate position.

In group A tuberculin sensitivity rose from 1:100 to 1:10,000 or 1:100,000 5 months after infection and remained at this level till death. In group D all animals were sensitive to 1:1000 6 weeks after infection; three died within the next month, but the remaining three rose to 1:10,000 $2\frac{1}{2}$ months after infection, and to 1:100,000 in $3\frac{1}{2}$ months. The average survival time was longest in groups B and C and least in group D. The differences between groups B and C on the one hand and group D on the other approach significance. At necropsy the lesions were least severe and extensive in group B; between the other three groups there was little difference.

There was no great difference between the four groups in the number of colonies of tubercle bacilli cultivated from the lungs *post mortem*.

In this experiment the number of control animals was rather small and the results are therefore difficult to assess. On the whole the suggestion is that animals kept continuously desensitised after infection develop a less rapid and severe type of disease than normal animals.

Experiment 3

2.8.35. Groups A and B were sensitised with human tubercle bacilli killed by heating at 100° C. for one hour on two successive days. They received a total dosage of 2.3 mg. in two injections. Two months after the first inoculation most of the animals reacted to the intradermal injection of 1:100 tuberculin.

30.10.35. Group A was left untreated. Group B and a new group C of normal animals were given 13 inoculations of O.T. within about a fortnight, the dose rising from 0.01 to 0.1 c.c. After 3 weeks the animals in group B were almost completely desensitised, while those in group A reacted to 1:1000 tuberculin.

11.11.35. The animals were infected, along with two new groups D and E, with approximately 90 living organisms.

After infection the animals in groups A, B, C and E were left untreated while those in group D were kept continuously desensitised to 1:10 tuberculin by subcutaneous injections of 0.1-0.2 c.c. of O.T. five times a week.

TABLE III
Desensitisation and infection in experimental tuberculosis in the guinea-pig (expt. 3)

Group	Total dosage of killed tubercle bacilli		No. of animals infected	Average dosage of O.T. (c.c.)		Tuberculin sensitivity		Survival time in days		Extent and severity of lesions post mortem				Colonies of T.B. in lung cultures		
	Amount	No. of doses		Before Infection	After Infection	Before Infection	After Infection	A.M.	S.D.	+++	++	+	0	Minimum	Maximum	G.M.
A	2.3 mg.	2	10	1:1000	Rose to 1:10,000 or 1:100,000	103.3	37.8	4	4	2	0	150,000	518×10^3	9×10^5
B	2.3 mg.	2	10	0.85	...	<1:10	Rose to 1:1000 or 1:10,000	105.0	38.5	2	6	1	1*	0*	250×10^3	1.2×10^5
C	10	0.85	Rose to 1:10,000 or 1:100,000	109.0	22.4	4	6	0	0	1.7×10^5	250×10^3	36×10^5
D	10	...	14.2	111.7	58.9	0	6	4	0	12,000	104×10^3	9×10^5
E	10	Rose to 1:10,000 or 1:100,000	94.5	24.3	2	7	1	0	100,000	1750×10^3	5.6×10^5

* Died 14 days after infection with no visible lesions

Between groups	A and D	A and E	D and E
Observed difference in mean survival time in days .	8.4	8.8	17.2
Standard error of difference	23.3	15.0	21.2
Observed difference divided by its standard error .	0.36	0.59	0.81

The main results of this experiment are recorded in table III

The rate of enlargement of the inguinal glands was much the same in all five groups

In group A tuberculin sensitivity rose from 1 1000 to 1 10,000 or 1 100,000 3 months after infection In group B sensitivity was re established to 1 10 within a fortnight of infection and rose to 1 1000 or 1 10,000 3 months after infection In group C sensitivity was established to 1 10 within a fortnight, and rose to 1 10,000 or 1 100,000 within 3 months Group E behaved like group C but most of the animals died before they reached the 1 100,000 level

The average survival time was longest in group D and shortest in group E but the difference fell far short of significance At necropsy the lesions were least severe and extensive in group D Between the other four groups the differences were not very striking The mean number of colonies of tubercle bacilli cultivated from the lungs *post mortem* was lowest in group B and highest in group C Between the other three groups there was little difference

The results of this experiment are in general harmony with the conclusions drawn from the two previous experiments They suggest that continuous desensitisation after infection tends to limit the progress of experimental tuberculosis Whether tuberculin treatment before infection is of any value it is difficult to say Group B differed very slightly from group A and group C from group E

Experiment 4

In this experiment an attempt was made to find out how far the effect of desensitisation is specific

4 8 37 Groups A, B and C were sensitised with dead tubercle bacilli Three doses were given at intervals of 4 weeks The first dose consisted of 1 mg of bovine bacilli which had been in contact with 0.5 c.c. of phenolised saline for three years the second and third of 2.5 and 1.0 mg respectively of human or bovine bacilli killed by exposure to 100° C for one hour A month after the last dose most of the animals reacted to 1 100 O.T. intradermally

2 11 37 Group A was left untreated Group B was given 31 doses of O.T. during the next 6 weeks starting with 0.001 c.c. and ending with 0.1 0.2 c.c. Group C was given 31 doses of 5 per cent glycerol broth which had been concentrated in a water bath to one tenth of its original bulk so as to render it similar in viscosity to O.T. The doses rose from 0.01 to 0.1 c.c. Larger doses were tried in the middle of the course but had to be abandoned owing to the amount of irritation that resulted At the end of this six weeks course of the 29 untreated allergic animals in group A, 1 reacted to 1 10, 17 to 1 100, and 11 to 1 1000 tuberculin Of the 30 animals in group B all but 5 were negative to 1 10 tuberculin of the 30 in group C 29 reacted to 1 10 and 1 to 1 100 tuberculin In other words the animals in group B were almost completely and those in group C partly desensitised to the intradermal injection of 1 10 tuberculin

15.12.37. All animals were inoculated intramuscularly into the thigh, together with 31 control animals in group D, with approximately 200 living organisms of a virulent human strain of tubercle bacilli.

After infection group B received subcutaneous injections of O.T., and group C subcutaneous injections of concentrated glycerol broth. These were given 6 days a week, the doses in both cases rising from 0.1-0.2 c.c. up to 0.3-0.4 c.c. a day. Some of the animals in both groups then began to lose weight, and the doses were reduced to 0.1-0.2 c.c. a day. After a further two months of this treatment the animals were weighed twice weekly and the dosage continued unless the animal had lost more than 30 g. since the previous weighing.

All animals were left till they died. *Post mortem*, a very careful examination, both macroscopic and microscopic, was made of each of the glands and viscera. The results were recorded in a series of index numbers. Eleven animals died after a pleuropneumonia infection due to an organism identified by Dr W. M. Scott as *Str. pneumoniae* type XIX, 4 in group A, 0 in group B, 5 in group C and 2 in group D. Three died shortly after infection from an unknown cause, and four from accidental causes. All these animals have been excluded from the results, which are summarised in table IV. Five animals in group B which apparently died as the result of an overdose of tuberculin have been included.

In group A the sensitivity to tuberculin rose gradually for 16 weeks after infection, when of 26 survivors 2 reacted to 1:100, 18 to 1:1000, and 6 to 1:10,000. In group B the majority regained some degree of sensitivity in spite of their daily injections with tuberculin; after 16 weeks, of 28 survivors 4 were still negative to 1:10, 17 reacted to 1:10 and 7 to 1:100; after 30 weeks 2 of the 18 survivors reacted to 1:1000. In group C the rise in sensitivity was still greater; after 16 weeks, of 28 survivors 2 reacted to 1:10, 7 to 1:100 and 19 to 1:1000, while after 25 weeks 5 out of 19 reacted to 1:10,000. In group D sensitivity became established fairly soon after infection and rose gradually till after 16 weeks, of 29 animals 1 reacted to 1:10, 4 to 1:100, 19 to 1:1000, 3 to 1:10,000 and 2 to 1:100,000; the maximum was reached after 25 weeks, when all reacted to 1:100-1:100,000. It will thus be seen that the controls in group D were the most sensitive and those injected with O.T. in group B the least sensitive. The average sensitivity in group C was definitely less than that in group A, showing that the daily injections of concentrated glycerol broth produced some desensitisation, but less than that produced by O.T. In all groups, particularly in A, C and D, there was a tendency for the sensitivity to fall in the later stages of the disease. It may be noted that on three separate occasions after infection the sensitivity of the animals in group C was tested to 1:10 concentrated glycerol broth with negative results; even animals reacting to 1:1000 tuberculin failed to respond to 1:10 glycerol broth, suggesting that non-specific desensitisation was more or less complete.

TABLE IV
Desensitisation and infection in experimental tuberculosis in the guinea pig (expt 4)

Group	Total dosage of killed tubercle bacilli		No of animals infected	Average dosage of O.T. or glycerol broth (c.c)		Tuberculin sensitivity		Survival time In days		Above extent and severity of lesions post mortem Below no of acid fast bacilli micro coagally						Colonies of T.B in lung cultures		
				Before infection	After infection	Before infection	After infection	A.M	S.D	++++	+++	++	+	0	Minimum	Maximum	O.M	
A	4.5 mg	3	29			1 100 or 1 1000	Rose to 1 1000 or 1 10,000	179.9	33.8	4 0	12 0	6 6	0 6	0 16	0	760,000	3100 × 10 ⁶	22.4 × 10 ⁶
B	4.5 mg	3	30	3.81 O.T	31.3 O.T	<1 10	Rose to 1 10 or 1 100	199.2	57.8	0 0	6 0	18 3	4 4	19 2	0	0	25,000 × 10 ⁶	590,000
C	4.5 mg	3	30	5.71 G.B	32.2 G.B	1 10	Rose to 1 100 or 1 10,000	192.9*	46.9	1 1	12 0	8 10	1 10	1 2	0	0	1030 × 10 ⁶	4.7 × 10 ⁶ *
D			31		.		Rose to 1 100 or 1 100,000	149.5	46.9	2 0	18 2	8 14	0 11	0 1	0	790,000	1520 × 10 ⁶	34.5 × 10 ⁶

* One animal destroyed by mistake before post-mortem

Animals which died from pleuropneumonia after infection or from accidental causes have been excluded. The number available for analysis of survival time was 22 in group A, 29 in group B, 23 in group C and 28 in group D.

	Between groups					
	A and B	A and C	A and D	B and C	B and D	C and D
	19.3	13.0	30.4	6.3	49.7	43.1
Observed difference in mean survival time in days	12.9	12.1	11.4	14.5	13.9	13.2
Standard error of difference	1.5	1.1	2.7	0.4	3.6	3.3
Observed difference divided by its standard error						

The average survival time was in inverse proportion to the degree of skin sensitivity to tuberculin at the time of infection. The differences between groups B and D and groups C and D may be regarded as significant. The last animal to die in group B survived for 341 days after infection. One animal in group C which died after 262 days showed no evidence of tuberculosis.

The macroscopic lesions *post mortem* were least extensive and severe in group B. Between the other three groups there was little difference. The lessened severity and extent of the lesions in group B seemed to affect all organs more or less equally. In the control group D the non-pulmonary tissues tended to be affected more severely than in the other three groups. Probably for this reason the control animals died earlier and their lung lesions were on the whole less developed than those in groups A and C. The number of acid-fast bacilli per microscope field in the various organs did not differ greatly between the four groups.

The average number of colonies of tubercle bacilli growing from the lungs varied in inverse proportion to the degree of sensitivity of the animals. It was considerably less in groups B and C than in the allergic and control groups A and D. It is interesting that whereas in groups A and D tubercle bacilli were cultivated from the lungs of every animal, in group B 6 and in group C 2 animals had negative lung cultures in spite of the fact that macroscopic lesions were present. This may have been due to some technical error, though there is no reason to suppose that it was.

The average weight of the animals in the different groups at the time of death was less in groups B and C than in groups A and D. Between the time of infection and the time of death the average loss in weight was as follows: group A 76 g., group B 104 g., group C 110 g. and group D 86 g. As previous workers have found, the process of specific or non-specific desensitisation affects unfavourably the general condition of the animal.

The results of this experiment seem to show that animals kept more or less desensitised with O.T. have a significantly longer survival time and develop less severe tissue lesions than control animals. Since animals prevented from reaching their full degree of sensitivity by treatment with concentrated glycerol broth likewise have a significantly longer survival time than controls, there is a strong suggestion that part at any rate of the desensitisation effect is non-specific.

DISCUSSION

Taken as a whole these experiments leave little doubt that guinea-pigs rendered allergic by vaccination with heat-killed tubercle bacilli, desensitised by large doses of tuberculin, infected

intramuscularly with very small numbers of virulent human tubercle bacilli, and kept more or less desensitised after infection by large doses of tuberculin, survive longer and have fewer lesions *post mortem* than allergic non-desensitised animals.

These results, though perhaps rather more definite, are in general consonance with those described by such workers as Rothschild, Friedenwald, and Bernstein (1934), Derick, Branch and Crano (1935), Branch and Kropp (1937), Higginbotham (1937), Follis (1938 *a* and *b*), Balteanu, Toma and Garaguli (1938, 1939), and Saenz (1939). Not all workers, however, agree on the beneficial effect of desensitisation. Willis and Woodruff (1938), and Willis, Woodruff, Kelly and Voldrich (1938), for example, found that their desensitised animals died before those in their non-desensitised group, and suffered from extensive bronchopneumonia. Possibly the magnitude of the dose of bacilli with which the animals were infected may have been to some extent responsible for this difference.

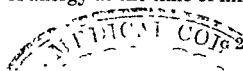
Before discussing the interpretation of the desensitisation type of experiment it may be well to analyse our own data more closely in order to throw some light on the importance of the allergic response in determining resistance to infection.

Relation between degree of sensitivity to tuberculin at the time of infection and survival time

Owing to the comparatively small number of animals in the four experiments described in this paper, we have included two others already reported by Schwabacher and Wilson (1938) in which guinea-pigs were vaccinated with living B.C.G. and subsequently infected with small doses of virulent human or bovine tubercle bacilli.

Table V shows the relation between the degree of skin sensitivity to tuberculin at the time of infection and the average survival time in days.

The results are very striking. In the allergic group the average survival time is inversely proportional to the degree of sensitivity at the time of infection. In two instances the differences between the sub-groups are statistically significant. In the animals of the control group, which were all negative to tuberculin at the time of infection, the average survival time was slightly longer than that of the 1:1000 allergic group and considerably longer than that of the 1:100,000 allergic group. On the other hand it was much shorter than in either the 1:10 or the 1:100 allergic groups. The suggestion is that (1) a slight to moderate degree of allergy at the time of infection is favourable to survival, (2) a high degree of allergy at the time of infection is definitely unfavourable to survival and (3) a very high degree of allergy at the time of infection is worse than no allergy at all.



These results are in accordance with those of Seibert (1932-33), who found that guinea-pigs highly sensitised by injections of the tuberculo-protein fraction (T.P.T.) tended to die sooner after infection than control animals. They also seem to agree with the results described by Sewall,

TABLE V

Degree of skin sensitivity to tuberculin at time of infection in relation to survival time (expts. 1-4 and B.C.G. 2 and 3)*

Group	Skin sensitivity at time of infection	No. of animals	A.M. survival time (days)	S.E. of A.M.
Control . .	0	77	144.9	6.18
Allergic . .	1 : 10	17	250.1	23.1
" . .	1 : 100	44	202.1	11.7
" . .	1 : 1000	19	132.8	13.5
" . .	1 : 100,000	10	98.7	22.8

A.M. = arithmetic mean S.E. = standard error (tables V and VI).

	Between groups		
	1 : 10 and 1 : 100	1 : 10 and 1 : 1000	1 : 10 and 1 : 100,000
Observed difference in mean survival time in days	48.0	117.3	151.4
Standard error of this difference	25.9	26.7	32.4
Observed difference divided by its standard error	1.9	4.4	4.7

* Schwabacher and Wilson, 1938.

de Savitsch and Butler (1934) who, by superinfecting guinea-pigs at varying times after a mild first infection, obtained evidence that immunity was more or less inversely proportional to the degree of skin hypersensitiveness at the time of the superinfecting dose.

Relation between degree of sensitivity to tuberculin reached after infection and survival time

Table VI shows the average survival time in relation to the highest degree of skin sensitivity reached after infection.

Here the results are less precise. In the control group there is a direct relationship between the average survival time and the highest degree of sensitivity reached but the differences are barely significant. In the initially allergic group there is the same general tendency, but the 1 : 100,000 group forms an exception to the rule. It is worth noticing that (1) the proportion of animals reaching a high degree of sensitivity after infection was greater in the control than in the initially allergic group and that (2) the average survival time of animals reaching a similar degree of sensitivity was greater in the initially allergic than in the control

group. Whether the average survival time increases because the degree of sensitivity continues to rise or the degree of sensitivity continues to rise because the animal goes on living it is impossible

TABLE VI

Highest degree of skin sensitivity reached after infection in relation to survival time (expts. 1-4 and B.C.G. 2 and 3)

Allergic				Control			
Highest sensitivity reached	No. of animals.*	A.M. survival time (days)	S.E. of A.M.	Highest sensitivity reached	No. of animals	A.M. survival time (days)	S.E. of A.M.
1 : 100	1	37.0	...	1 : 100	1	89	...
1 : 1000	12	147.3	21.5	1 : 1000	19	116.3	12.4
1 : 10,000	50	212.8	11.1	1 : 10,000	19	147.7	15.8
1 : 100,000	16	109.4	8.1	1 : 100,000	38	159.3	9.3
	79				77		

	Between groups		
	Allergic	Controls	
	1 : 1000 and 1 : 10,000	1 : 1000 and 1 : 10,000	1 : 1000 and 1 : 100,000
Observed difference in mean survival time in days	65.5	30.4	42.0
Standard error of difference	24.2	20.1	15.6
Observed difference divided by its standard error	2.7	1.5	2.7

* Two animals omitted, one (died in 49 days) because it was not tested after infection, the other (died in 36 days) because its sensitivity fell after infection. Allergic but not control animals in expt. 1 (table I, group A) also excluded because their sensitivity fell after infection.

to say. The fact however that in expt. 1, in which the sensitivity of the allergic animals fell after infection, the average survival time was considerably less than in the corresponding control animals, suggests that the former explanation is more likely to be correct.

On Rich's hypothesis that allergy plays no part in immunity and is indeed actively harmful, these results are difficult to explain. They suggest rather that the ability to develop a good allergic response to infection is of survival value to the host and that the failure to do this, as in expt. 1, is an indication of poor resisting powers.

Relation between degree of sensitivity to tuberculin and amount of caseation found post mortem

Finally we have attempted to find out whether there is any relation between the degree of sensitivity to tuberculin at the time of infection or the degree of sensitivity reached after infection

and the amount of caseation found in the lesions *post mortem*. In estimating this we have assessed the amount of caseation present in the femoral *plus* inguinal, the sublumbar and the portal glands, and in the spleen and liver, by a series of index numbers, and have added the results together for each animal separately. Unfortunately our records were complete enough to enable us to do this only in expt. 4. The figures, therefore, are very small and must be interpreted with the greatest caution. Since it may be argued that the longer an animal lives the more likely is caseation to go on progressing, we have included the average survival times (table VII).

TABLE VII

Relation between average degree of caseation found post mortem and (1) average sensitivity to tuberculin at time of infection, and (2) average highest degree of sensitivity reached after infection (expt. 4)

Skin sensitivity at time of infection	Group and no. of animals	Degree of caseation	A.S.T.
0	D (28)	5.9	149.5
<1 : 10	B (23)	3.5	194.0
1 : 10	{ A (1) B (5) C (22) } 28	4.0	198.1
1 : 100	{ A (11) C (1) } 12	4.1	193.2
1 : 1000	A (10)	5.9	165.3
Highest sensitivity reached			
1 : 10	B (13)	2.9	178.8
1 : 100	{ B (14) C (4) D (1) } 19	4.3	201.8
1 : 1000	{ A (7) B (1) C (14) D (15) } 37	5.8	165.4
1 : 10,000	{ A (15) C (5) D (8) } 28	5.3	181.6
1 : 100,000	D (4)	9.5	175.3

A.S.T. = arithmetic mean survival time in days.

Only 28 animals are included in group B, as one post-mortem examination was omitted in error.

On the whole it looks as if the degree of caseation varied more or less with the degree of sensitivity reached after infection. That this is not a simple function of survival time is fairly clear from the fact that the longest average survival time was recorded for the group which reached a sensitivity of only 1 : 100. So far as sensitivity before infection is concerned, the degree of caseation

appears to be least in the animals with a slight to moderate degree of allergy and greatest in those with no allergy at all or with a fairly high degree of allergy. This observation again cannot be explained on the basis of the average survival time, since the animals which lived longest showed the least degree of caseation.

Lurie (1934) reached the conclusion that caseation was to some extent a function of allergy. In so far as this relates to the degree of skin sensitivity reached after infection, our results afford some support to this conclusion.

Interpretation of the desensitisation type of experiment

Rich regards a sensitised animal that is treated with large doses of tuberculin till its skin becomes insensitive as no longer allergic. Finding that such an animal is as resistant to infection as a non-desensitised allergic animal, he concludes that allergy has nothing to do with immunity. There seem to us to be two fallacies in this argument.

The first is the assumption that the liberal dosage with tuberculin has no immunising effect. It is probably true, as Follis (1938a) points out and as our own experiments indicate, that inoculations of tuberculin into normal animals prior to infection do not increase their resistance. But inoculations of tuberculin into *allergic* animals may quite well set up a series of focal inflammatory reactions which may confer on the tissues some degree of resistance to the invasion of tubercle bacilli.

The second is the false assumption that desensitisation is complete merely because the skin reaction to tuberculin is reported as negative. In our own work we adopted a severe standard for a positive reaction—a standard that would correspond with a three-plus reaction of most American workers—and we found it very difficult to maintain our animals desensitised after infection. From a careful study of the records of previous workers it may be doubted whether anyone has yet succeeded in establishing and maintaining complete desensitisation without either killing a considerable proportion of the animals—sometimes as great as 92 per cent.—or so affecting their general health as to render them no longer strictly comparable with controls. The most, we believe, that can be achieved by large doses of tuberculin without damaging the health of the animals is to render them relatively insensitive to tuberculin, but never completely so. In other words it is a mistake to assume that a “desensitised” animal is completely non-allergic.

Even if complete desensitisation of the skin could be achieved and maintained it would still be dangerous, in view of Freund's (1929) demonstration that young tuberculous guinea-pigs are as

sensitive as adult guinea-pigs to the intraperitoneal inoculation of tuberculin but are practically insensitive to its intracutaneous injection, to conclude that the absence of a cutaneous reaction was proof of the concurrent absence of a systemic reaction.

As a corollary to this objection it may be pointed out that no amount of desensitisation seems capable of altering the fundamental allergic state of the animal. That this is true is shown by the rapid return of skin hypersensitiveness following the discontinuance of the daily injections of tuberculin. The "desensitised" state can persist only so long as there is an excess of antigen in the tissues. Tuberculin treatment may be regarded as symptomatic treatment which, acting like a cloak, serves to obscure the more blatant manifestations of the allergic state, but leaves unaffected the inherent ability of the tissues to respond abnormally. A similar conclusion has already been reached by Vallery-Radot, Mauric and Hugo (1936) in regard to sensitisation in general in the human subject.

Since the suppression of the exaggerated cellular response requires the daily administration of large quantities of tuberculin, it must be presumed that the tuberculin is reacting in some way with the cells. The effect of this must be to produce minor inflammatory reactions around each tuberculous focus in the body with the result that the dissemination of the bacilli is retarded and the rapid caseation which seems more liable to occur in the highly allergic animal is prevented. Such animals may be expected to survive longer on the average and to show less severe lesions at necropsy than control infected animals whose allergy has been allowed to develop to a point at which the very intensity of the reaction in the internal organs—particularly the lungs—proves prejudicial to life.

On the whole we believe that the various desensitisation experiments show not that allergy has nothing to do with immunity, but that, when properly controlled, it constitutes a very valuable mechanism in the defence of the body against the invasion of the tubercle bacillus. In this connection attention may be drawn to the peculiar behaviour of the group of allergic animals in our expt. 1. Contrary to what was observed in all the other experiments, the sensitivity of these animals fell after infection and the mean survival time was less than that of the control group. It is difficult to avoid the suggestion that allergy is in some way related to immunity.

Such an interpretation seems to be in general consonance with clinical findings in human beings. The work of Heimbeck (1927, 1932, 1933) and of numerous other observers suggests very strongly that adolescent and young adult persons possessing a moderate degree of allergy tend to be more resistant to the tubercle bacillus than persons who are non-allergic or only

very weakly allergic. Of particular significance is Heimbeck's finding that von Pirquet-negative nurses who were vaccinated with B.C.G. and who developed skin sensitivity as a result proved more resistant than similarly vaccinated nurses who remained persistently tuberculin negative.

The apparently greater tendency to develop tuberculosis after certain infectious fevers and after pregnancy may be of significance when it is realised that these abnormal states are often accompanied by a disappearance of allergy. The gradual loss of the allergic reaction is likewise characteristic of progressive tuberculosis in its later stages. It may be argued that in all these conditions the resistance of the patient is determined by humoral antibodies, and that the fluctuations in allergy are merely unimportant associated phenomena. It seems far more likely, however, that among the multiple factors involved in the defence of the host against the tubercle bacillus, a moderately increased degree of cellular reactivity plays a not unimportant part.

This conclusion is similar to that recently arrived at by Menkin (1938) who, after reviewing the whole subject of the part played by inflammation in immunity, expresses himself in the following terms (p. 397). "The available facts therefore strongly suggest that the allergic inflammation is quite likely one factor among several others which is involved in the manifestation of immunity in tuberculosis."

SUMMARY AND CONCLUSIONS

1. Guinea-pigs can be rendered sensitive in 2-6 months to 1:1000 or 1:10,000 tuberculin injected intradermally by inoculation of relatively small doses of heat-killed tubercle bacilli.

2. If the animals are left without further treatment their sensitivity wears off in the course of a few months.

3. By an intensive course of injections of Old Tuberculin they can be desensitised more or less completely, but if this is stopped sensitivity gradually returns and reaches its original level within a few weeks.

4. If desensitised animals are infected with virulent tubercle bacilli they may be kept desensitised by large daily doses of tuberculin, but it is doubtful whether complete desensitisation can be achieved or maintained without seriously impairing the health and life of the animals.

5. Desensitisation with tuberculin is mere symptomatic treatment; it appears to have no effect on the fundamental allergic state.

6. Under the conditions of our experiments animals rendered allergic by vaccination with heat-killed tubercle bacilli, desensitised by injections of tuberculin before infection and kept more or less

desensitised after infection had a rather longer average survival time and showed less severe lesions at necropsy than did vaccinated non-desensitised or non-vaccinated control animals.

7. Non-vaccinated animals which were prevented from becoming hypersensitive after infection reacted more favourably than non-vaccinated animals which were given tuberculin treatment only before infection, but the number of animals was too small to render the difference significant.

8. Partial desensitisation of vaccinated animals towards tuberculin could be achieved by injections of concentrated glycerol broth and the development of hypersensitiveness after infection could be partly controlled by daily injections of this substance.

9. Animals so desensitised had a rather longer average survival time and tended to show rather less severe lesions at necropsy than did vaccinated non-desensitised animals.

10. Analysis of experimental results showed that (a) in allergic animals the average survival time was inversely proportional to the degree of skin sensitivity at the time of infection, (b) in both allergic and normal animals the average survival time was more or less proportional to the degree of skin sensitivity reached after infection and (c) in both allergic and normal animals the degree of caseation observed *post mortem* tended to vary with the degree of skin sensitivity reached after infection.

11. It is suggested that "desensitisation" with tuberculin, which is probably never complete in the healthy animal, acts not by abolishing allergy but by converting a high into a low degree of allergy. The repeated doses of tuberculin set up minor inflammatory reactions around each tuberculous focus in the body and these tend to retard dissemination of the tubercle bacilli and to prevent the rapid and destructive caseation which seems more prone to occur in highly hypersensitive animals. This interpretation appears to be supported by histological observations kindly carried out for us by Professor G. R. Cameron, which it is hoped to report in a later paper.

12. The desensitisation type of experiment can be interpreted as showing either, as Rich believes, that allergy and immunity are causally unrelated phenomena or, as we prefer, that a controlled allergic response is of value in increasing resistance to tuberculosis. If allergy and immunity are to be dissociated some other less ambiguous type of experiment will have to be devised to prove it.

We are indebted to the Medical Research Council for financial assistance in this investigation. We should also like to express our thanks to Dr R. A. O'Brien of the Wellcome Physiological Research Laboratories, who placed at our disposal a generous supply of O.T. for use in expt. 4.

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THE EFFECT OF BLOOD SERUM ON THE GROWTH OF TUBERCLE BACILLI IN THE DEPTH OF LIQUID MEDIA

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IN 1932 Kallós and Nathan observed that the serum from patients with skin tuberculosis does not support the growth of tubercle bacilli *in vitro* in the same way as serum from normal persons and patients with pulmonary tuberculosis. Later I endeavoured to show (Pagel, 1934-35) that this applies also to the serum of cases with a pulmonary lesion accompanied by some extra-pulmonary manifestation (disseminated tuberculosis). In glycerol peptone broth no growth of tubercle bacilli occurred in the depth of the medium except after the addition of a certain quantity of human or animal serum, but the serum from cases of disseminated tuberculosis did not favour such growth.

Since then I have examined a further series of 210 cases by the same method. A saline suspension (0.2 c.c.) containing approximately 1 mg. of culture per c.c. was added to a 1 : 8 dilution of active serum in glycerol peptone broth (0.5 c.c. serum to 3.5 c.c. broth). After incubation at 37° C. for 10-20 days results were obtained as shown in table I. With most sera flocculent growth

TABLE I

Effect of serum from various sources on the growth of tubercle bacilli in glycerol-peptone broth and Kirchner's medium

Medium	Total cases	Source of sera permitting luxuriant growth			Source of sera falling to support luxuriant growth			Result in agreement with theory	Result unexpected
		Isolated pulmonary phthisis	Disseminated tuberculosis	Normal persons	Isolated pulmonary phthisis	Disseminated tuberculosis	Normal persons		
Glycerol peptone broth	210	99	20	14	18	58	1	171	30
Kirchner's medium	230	122	13	15	20	66	0	203	33

was observed, whereas in others none or only scanty growth was visible with the naked eye. Films, 0.5 cm. in diameter, made with one loopful of the contents of the tubes with scanty or no

growth showed individual bacilli in small numbers or one or two small clusters, whereas 5-20 large clusters were seen in films from tubes with luxuriant growth. A loopful of the former subcultured on Dorset's medium produced after 14 days 0-10 colonies as against 100-150 colonies appearing in a similar subculture from a tube with luxuriant growth.

Out of the 133 sera which supported growth, 113 were from normal persons or from patients with the common isolated pulmonary phthisis of the adult type, whereas in 20 cases the clinical and X-ray picture showed evidence of dissemination. Among the 77 sera in which bacillary growth was scanty or failed to appear, 58 were from cases with extrapulmonary tuberculosis or typical hæmatogenous spread in the lung, and 19 from ordinary phthisis. If, as suggested (Pagel, 1934-35), the test is used for a serological differentiation of isolated pulmonary phthisis from disseminated tuberculosis it failed in about 1 : 7 of the cases of isolated adult phthisis and in 1 : 4 of those with disseminated tuberculosis.

Kirchner's medium was used for the next series of experiments, as it eliminated the disadvantages associated with the use of glycerol peptone broth, such as the necessity of a careful control of the pH and the use of easily emulsifiable cultures of tubercle bacilli.

Kirchner's medium contains the following ingredients.

Disodium phosphate (Na_2HPO_4)	.	.	.	3.0 g.
Monopotassium phosphate (KH_2PO_4)	.	.	.	4.0 "
Magnesium sulphate	.	.	.	0.6 "
Sodium citrate	.	.	.	2.5 "
Asparagin	.	.	.	5.0 "
Glycerol	.	.	.	20.0 "
Distilled water	.	.	.	1000.0 c.c.

To 2.5 c.c. of sterilised medium were added 0.2 c.c. of the bacillary suspension and 2.5 c.c. of active serum and the tubes were incubated at 37° C. If growth occurred it was visible at the bottom of the tube or as an opacity throughout the medium after shaking at the end of 10-20 days' incubation. After a further 20 days the growth appeared on the surface of the fluid and extended to the wall of the tube. Growth was only rarely obtained in the basic medium alone without addition of serum and was scanty and very slow.

With this technique 236 cases were examined. The result was similar to that obtained with glycerol broth (table I). Out of 150 sera in which growth was luxuriant, 137 were from normal persons or from patients with isolated pulmonary phthisis and 13 from cases of disseminated tuberculosis. Among the 86 sera with scanty or no growth, 66 were from patients with disseminated tuberculosis and 20 from others. The test failed therefore in less than 1 : 10 of the former and in 1 : 4 of the latter group. The number of failures is greatly reduced when those cases are deducted

in which disseminated extrapulmonary lesions were latent or in which conditions such as amyloid disease, pleural effusion, advanced cachexia, etc. were present and growth unexpectedly failed in the serum. On the other hand the inactive character of an extrapulmonary lesion may account for luxuriant growth in the serum of some cases of disseminated tuberculosis (see table II).

Although the results are similar to those obtained with glycerol broth I can confirm the statement of Kallós and Nathan that Kirchner's medium is superior to broth for this purpose, as it yields consistent results with any strain of tubercle bacilli. It was therefore generally adopted in the following experiments.

The result of these 446 examinations confirms, therefore, those obtained previously in 70 cases (Pagel, 1934-35) in showing that sera from most cases of isolated phthisis of the adult type support the growth of tubercle bacilli *in vitro* in the same way as sera from normal individuals, whereas those from patients with disseminated tuberculosis often fail to do so.

The bearing of this phenomenon on our knowledge of the different immunological conditions underlying isolated pulmonary phthisis and disseminated tuberculosis has already been discussed (Pagel, 1936-37, 1939). The phenomenon may be explained by a change developing gradually in the serum after a hæmic metastasis. I have observed this several times. When the acute symptoms of a lesion in the epididymus or a new abscess in bone tuberculosis had subsided, serum which had previously allowed a luxuriant growth of the bacilli lost this power. This condition persists for a long period, whether the process is active or not. The result of the test does not necessarily depend upon the activity or the progressive character of the disease (see table II), but indicates whether it belongs to the group of disseminated or isolated pulmonary tuberculosis. Patients with advanced as well as those with moderate or slight degrees of disseminated tuberculosis yield sera in which bacillary growth is not well supported. As a rule, however, in the patients with disseminated tuberculosis most sera yielding scanty or no growth were from active cases (32/52) and most of those supporting growth from inactive cases (24/29).

TABLE II

Relation of activity of lesion to effect of serum on growth

Effect of serum on growth	Extrapulmonary lesion	
	active	inactive
Growth scanty or absent	32	20
Luxuriant growth	5	24

It should be emphasised in this connection that failure to support growth does not indicate any special individual or species resistance to tuberculosis. The serum of healthy individuals does not prevent or retard growth nor does the serum of various species with different natural resistance to tuberculosis. In the serum of animals with low susceptibility such as the horse or rat, growth is as luxuriant as in that of the ox, sheep or rabbit—animals with much lower resistance. The growth in guinea-pig serum is slightly more and in pig serum slightly less luxuriant, but the differences are small. It is therefore obvious that neither species nor individual resistance towards tuberculosis has any appreciable influence on the growth-supporting power of the serum. The observations of Courmont and Gardère (1933) that growth was bad in serum from animals with high natural resistance are not borne out by this investigation.

Through the courtesy of Dr Norah O'Leary I was able to examine the sera of 25 children, including cases of tuberculosis and rheumatism. The result is shown in table III. There were 10 sera which failed to support growth, most of them from cases of disseminated and active tuberculosis of the abdominal or hilar lymph glands: 15 which supported growth were from patients with chronic isolated pulmonary tuberculosis of adult type (4) or from normal or rheumatic children (11).

TABLE III

Effect of sera of children on growth of tubercle bacilli

Source of sera	Growth of tubercle bacilli		Total sera
	Luxuriant	Scanty or absent	
Pleurisy with effusion	1	2	3
Disseminated tuberculosis (spread in lungs and extrapulmonary condition)	0	4	4
Active tuberculosis of hilar or abdominal glands	0	3	3
Chronic isolated pulmonary tuberculosis with or without cavitation	4	0	4
Healed cases or no definite lesion or rheumatism	10	1	11

To determine whether the sera which failed to support growth were affected by heating, an experiment was made simultaneously with active and inactivated portions, the latter being heated for $\frac{1}{2}$ hour at 56° C. in the water-bath. The results are shown in table IV. In most cases no better growth was obtained after inactivation. On the other hand growth-supporting sera were only exceptionally affected by inactivation.

TABLE IV

Effect of inactivation of serum at 56° C for 30 minutes

	Active serum	Inactivated serum
Growth luxuriant	16	14
Growth scanty or absent	11	0
Growth more luxuriant after inactivation		2
Growth less luxuriant after inactivation		2

Although without influence on the intensity, inactivation did affect the type of growth in the mixture of serum and Kirchner's medium. In tubes containing active serum, growth was visible as coarse flocculi and smears showed clumps of bacilli. With inactivated serum it appeared as a fine homogeneous suspension and no clumps were found in smears.

The question as to whether the factor responsible for the scarcity or absence of growth was of the nature of an antibody was approached by means of absorption experiments.

Five c.c. of serum were divided into 2 equal portions. One was used untreated and the other was mixed with a milky suspension of organisms killed by heating for half an hour at 60° C in a water bath. The organisms used were *Bact. coli*, staphylococci, diphtheria bacilli and 5 members of the acid fast group—virulent human tubercle bacilli from sputum, avian bacilli, snake bacilli, smegma bacilli and acid fast bacilli obtained from normal guinea pigs in the Pasteur Institute, Paris, the so called "BCG S" strain. The serum was left in contact with the killed bacilli for 3 hours at 37° C and then centrifuged at approximately 3000 r.p.m. for about 10 minutes.

The results of the absorption experiments are recorded in tables V and VI.

TABLE V

Effect of absorption of sera with various organisms on their power to support growth of tubercle bacilli

	No. of sera showing growth before and after absorption with									
	human tubercle bacilli		avian tubercle bacilli		snake bacilli		smegma bacilli		BCG S	
	Non absorbed	Absorbed	Non absorbed	Absorbed	Non absorbed	Absorbed	Non absorbed	Absorbed	Non absorbed	Absorbed
Growth luxuriant	60	59	3	3	6	6	40	39	6	6
Growth scanty or absent	15	5	8	2	11	0	40	12	5	5
Support of growth induced by absorption		10		0		11		38		0
Support of growth abolished by absorption		1		0		0		1		0

TABLE VI

Effect of absorption with staphylococci, diphtheria bacilli and Bact. coli

	Sera giving growth before and after absorption with					
	staphylococci		diphtheria bacilli		<i>Bact. coli</i>	
	Non-absorbed	Absorbed	Non-absorbed	Absorbed	Non-absorbed	Absorbed
Growth luxuriant . . .	7	6	12	12	16	14
Growth scanty or absent	20	17	6	5	13	4
Support of growth induced by absorption	...	3	...	1	...	9
Support of growth abolished by absorption	...	1	...	0	...	2

It is seen in table V that absorption with killed bacilli of the human, avian, and particularly of the snake and smegma type converted a serum which failed to support the growth of human tubercle bacilli into one yielding luxuriant growth, whereas the so-called BCG-S failed to do so. In the absorbed sera the growth of avian strains and of the smegma bacillus and BCG-S was also increased.

Absorption with staphylococci and diphtheria bacilli had this effect only exceptionally (table VI). Absorption with *Bact. coli* increased the power to support growth in 9 out of 13 sera; in 2 it had the opposite effect, luxuriant growth appearing in the non-absorbed serum but not in the absorbed.

The possibility that the effect of absorption was due to an action on complement was ruled out, as sera inactivated for $\frac{1}{2}$ an hour at 56° C. were affected in the same way as active sera.

There are two points against the assumption that specific antibodies are responsible for the effect of sera from patients with disseminated tuberculosis.

(a) Absorption with heat-killed bacilli causes some acceleration and increase of growth in growth-supporting sera from normal men or animals.

(b) Tuberculous infection or vaccination with heat-killed tubercle bacilli or living acid-fast organisms does not alter the growth-supporting power of the serum of guinea-pigs or rabbits (table VII).

It seemed possible that the killed bacilli added to the serum in the absorption tests had acted as a growth-promoting factor by the liberation of lytic products, as suggested by Gordon and Hoyle (1936) in explanation of certain phenomena observed in absorption experiments on the general bactericidal action of normal serum. To see if the addition of killed acid-fast bacilli to a fluid culture medium increased the growth of tubercle bacilli, experiments

were made with the 5 per cent. egg-yolk water described by Weise (1925) and Weise and Fernbach (1925). If a suspension of tubercle bacilli as used above is added to this medium, luxuriant and flocculent growth will follow in about 4-8 days, very similar to that obtained in mixtures of serum and Kirchner's medium. If, however, the egg-yolk medium is treated with killed acid-fast bacilli in the same way as in the absorption experiments, there is no alteration of the growth. If "absorption" is continued for

TABLE VII

Effect of serum from normal, infected and vaccinated animals on the growth of tubercle bacilli

Source of serum	Number of sera inducing		Total
	luxuriant growth	scanty or no growth	
Guinea-pigs—			
(a) normal	10	1	11
(b) tuberculous	4	1	5
(c) vaccinated *	10	0	10
(d) vaccinated * and infected	5	0	5
Rabbits—			
(a) normal	5	0	5
(b) tuberculous	5	0	5
(c) vaccinated †	6	1	7
(d) vaccinated † and infected	5	1	6
Pigs (normal)	6	2	8
Sheep (normal)	5	0	5
Oxen (normal)	4	0	4
Horses (normal)	7	2	9
Rats (normal)	4	0	4

* 5-10 weekly injections of heat-killed tubercle bacilli intraperitoneally.

† 5-10 weekly injections of living avirulent acid fast bacilli intravenously.

12 hours the growth is not only not increased but is even more scanty than in the untreated tubes. A growth-promoting action of the heat-killed bacilli was therefore not demonstrable under conditions very similar to those in the above experiments. Nor could any promotion of the surface growth of tubercle bacilli be obtained on glycerol peptone broth or synthetic media such as those of Lockemann or Long after treatment with killed smegma bacilli for 3-24 hours at 37° C.

No evidence could be adduced therefore for the theory that by adding heat-killed bacilli to the serum a growth-promoting factor had been introduced and simulated "absorption." It is more likely that absorption of a growth-inhibiting factor has taken place, although the range of absorbing organisms is too wide to allow of the assumption of the intervention of a specific antibody.

Finally an attempt was made to obtain direct evidence that a growth-inhibiting factor was responsible for the sparse growth of tubercle bacilli in serum from patients with disseminated tuberculosis. For this purpose a mixture of 2.5 c.c. of active serum and 2.5 c.c. of Weise's medium was inoculated with 0.2 c.c. of bacillary suspension as used in the previous experiments. The growth was compared with that obtained in the same serum mixed with Kirchner's medium and in 2.5 c.c. of Weise's medium without addition of serum but diluted with 2.5 c.c. of saline. The experiment was made with 5 sera and the growth recorded after 18 days. With 3 sera sparse and with 2 sera luxuriant growth was obtained on Kirchner's medium. No growth at all was observed in Weise's medium to which the 3 former sera had been added. With the 2 others the growth was as luxuriant as on Kirchner's medium. Simple dilution with saline had no effect on the growth in Weise's medium. It is therefore obvious that 3 of the sera contained a factor which actively inhibited bacillary growth. These had been taken from patients with active disseminated tuberculosis.

Summary

1. About 90 per cent. of sera from normal persons and patients with bronchogenic phthisis restricted to the lungs support luxuriant growth of tubercle bacilli in the depths of glycerol peptone water or Kirchner's medium, whereas approximately 70 per cent. of the sera from patients with chronic disseminated tuberculosis fail to do so.

2. The failure to support growth is more pronounced in sera from active than in those from inactive cases, but occurs irrespective of the activity of the patient's lesion.

3. There is no parallelism between the intensity of growth in serum and individual or species resistance to tuberculosis.

4. The effect of serum on the luxuriance of growth is not influenced by inactivation though the type of growth may be.

5. Power to support luxuriant growth can be induced in a serum which has failed to do so by absorption with acid-fast organisms or with *Bact. coli*, but not with staphylococci or diphtheria bacilli. The effect is not due to the introduction of a growth-promoting factor, or to an action upon complement. Absorption of normal human serum slightly increases its growth-supporting power.

6. Vaccination of rabbits and guinea-pigs with heat-killed tubercle bacilli does not affect the growth-promoting power of their sera.

7. Absence of bacillary growth or scanty growth is due to the presence of a growth-inhibiting factor.

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ADAPTATION IN VEINS TO INCREASED INTRA- VENOUS PRESSURE, WITH SPECIAL REFERENCE TO THE PORTAL SYSTEM AND INFERIOR VENA CAVA¹

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(PLATES XVI XVIII)

THE venous system has received little attention from pathologists in the past. Endophlebitis and phlebosclerosis, it is true, are recognised but their definition is obscure and there is much to be found out about their pathogenesis and cause. The structure of veins presents regional differences and it might reasonably be assumed that such differences would be found, too, under pathological conditions. In the investigation now to be described, attention has been confined to two sets of veins, the portal system and the inferior vena cava, and one pathological condition, increase of venous pressure.

Material and methods

Material and methods Random samples of the extra hepatic portion of the portal vein, the mesenteric and splenic veins and the inferior vena cava were collected from 54 cases † *post mortem* and fixed in 10 per cent formalin. They were examined in the gross after staining with scarlet red for fatty deposits in the intima. Some were then embedded in gelatin and frozen sections of these were again stained for fat. Because no fat was ever demonstrated by either procedure, double embedding in celloidin and paraffin was substituted. Six to eight blocks were made from each vein and both longitudinal and transverse sections were cut at $7\ \mu$ and stained with Ehrlich's acid hematoxylin and eosin, orcein for elastic and Heidenham's azan stain for connective tissue and muscle.

Measurements With normal veins the thickness of the intima is negligible, therefore measurements of this layer were not made. The media was measured from the internal elastic lamina to the outer edge of the circular muscle, the adventitia from the edge of the circular muscle to the

* Part of a thesis approved for the degree of Doctor of Philosophy in the University of London.

† The complete details of the measurements, etc., are not given in this communication for reasons of economy of space but are included in the original thesis.

outer edge of the longitudinal muscle. The former was measured under high power magnification, the latter under low power, using a micrometer eyepiece. Numerous measurements were made from each vessel and the average recorded. Care was taken to avoid measurements from parts of the sections which had been cut obliquely.

RESULTS

Macroscopic examination

Gross examination of the portal, mesenteric and splenic veins as a rule revealed no outstanding intimal change. The inferior vena cava sometimes showed intimal thickening. At the junction of the splenic and portal veins there was often seen a slightly elevated ridge, more marked at the lower border of the junction. I have called this the "splenic sphincter." It is not merely a fold in the intima, *i.e.* a rudimentary valve, but contains circular muscle fibres beneath the intima, seen in the gross specimen with the aid of the low power dissecting microscope as well as in sections prepared from this region (figs. 1 and 2). A similar ridge was not found at the junction of the mesenteric and portal veins.

Normal histology

Pathological changes were not met with in half the cases studied. Those showing no apparent change and collected from cases without a history of cardiac or hepatic disease were taken as normal controls and form the basis for the following description.

1. *Portal vein* (fig. 3). In the adult the portal vein has three coats—intima, media and adventitia. The intima consists of a very thin endothelial lining, some subendothelial connective tissue and a definite internal elastic membrane. In the subendothelial connective tissue longitudinal muscle fibres are not obvious under normal conditions. The internal elastic lamina is actually a membrane composed of thick and thin elastic fibres interlacing in longitudinal, circular and oblique directions. The media is well developed and comprises about one-fifth of the total width. It contains circular muscle fibres arranged singly or in very thin strands of 2-4 fibres. There is a small amount of intervening connective tissue. The adventitia contains a large number of closely packed longitudinal muscle bundles. Between the media and adventitia there is frequently a connective tissue zone which may contain a few longitudinal or circular muscle fibres. In the measurements taken, this zone is included in the adventitia. The media is especially rich in elastic fibres, which are mainly circular in direction. The adventitia has fewer elastic fibres, which are longitudinal in direction. There is an external elastic membrane rich in longitudinal elastic fibres.

ADAPTATION TO INCREASED INTRAVENOUS PRESSURE



FIG. 1.

FIG. 2.



FIGS 1 and 2—Longitudinal sections showing the ridge or "splenic sphincter" at the mouth of the splenic vein H. and E.



FIG 3—Normal portal vein in longitudinal section, showing very thin intima and thin strands of muscle fibres in the media H. and E.

2. *Mesenteric vein.* The mesenteric vein is a direct continuation of the portal vein and has the same general pattern, though its wall contains more circular and less longitudinal muscle the further away it is from the main portal trunk. The distribution of the elastic tissue is similar to that in the portal vein.

3. *Splenic vein.* The calibre of the splenic vein is smaller than that of the portal and mesenteric veins but its media contains as much circular muscle. The adventitia is much thinner and contains fewer longitudinal muscle fibres. The distribution of the elastic tissue remains the same.

The "splenic sphincter." Beneath the ridge at the mouth of the splenic vein the muscle in the media becomes dispersed and is 3-4 times as thick as the media elsewhere (figs. 1 and 2). In view of the fact that at the mouth of the hepatic vein there is a similar muscle band which has been named by Dale and his associates the "hepatic sphincter" (Bauer *et al.*, 1932) and at the mouth of renal vein a similar muscle band has been regarded as a sphincter by Kampmeier and Birch (1926-27), I am inclined to regard this splenic thickening also as a sphincter.

4. *Inferior vena cava (fig. 7).*—The inferior vena cava presents certain structural peculiarities. It is a much thicker vessel than the portal vein. The intima and subendothelial connective tissue and the internal elastic lamina are similar to those of the portal vessels. In the subendothelial connective tissue there are no obvious longitudinal muscle fibres under normal conditions. The media is not prominent and the adventitia predominates over the media. The circular muscle in the media is composed of single fibres or thin-strands of 2-4 fibres concentrated in a zone next to the internal elastic lamina. Further away the muscle fibres are somewhat dispersed and separated by collagen fibres. The circular muscle increases in amount distally. In other words the circular muscle seems to decrease as the vessel approaches the heart. The adventitia forms the principal part of the vessel and contains an abundance of very thick bundles of longitudinal muscle closely packed together with a small amount of intervening connective tissue. Between the media and adventitia there is usually a connective tissue zone containing sparsely scattered thin strands of longitudinal muscle and sometimes a few circular muscle fibres. In the measurements taken, this zone is included in the adventitia. Elastic fibres are evenly distributed throughout the whole wall. Thus the distribution of elastic fibres in the inferior vena cava is different from that of the portal vein and its main tributaries, where the elastic tissue is mainly located in the media. In six of this group of normal controls the inferior vena cava showed unusual changes which are described later.

Pathological histology

Atherosclerosis, scarring of the media and inflammatory reactions were never encountered in the portal vein or its tributaries. A frequent finding was thickening of the intima with the development of well defined longitudinal muscle fibres in the subendothelial connective tissue and muscular hypertrophy of the media. This was seen only in cases of cardiac failure, emphysema and cirrhosis of liver, *i.e.* when the venous pressure was increased. In the *portal vein* the intimal thickening is generalised but not uniform, being greater in some places than in others. This gives the impression that changes start as multiple foci. Beneath the intima there are thin strands of longitudinal muscle fibres (figs. 4 and 5). The circular muscle of the media is hypertrophied, the muscle fibres forming groups of thicker strands of 15-25 fibres instead of the normal thin strands of 2-4 fibres. The adventitia shows no appreciable change. As a rule there is no duplication of the internal elastic lamina and the normal pattern and distribution of elastic tissue are still preserved.

The muscular hypertrophy of the media is not influenced by age. Most of the specimens studied were from elderly subjects and in these the structure of the portal vein did not show much deviation from that of the young subjects. The decrease of muscle and elastic tissue described by Neumann (1937) in the ageing great saphenous vein were not seen in the portal vein.

In a few instances similar changes were observed in the *mesenteric* and *splenic veins* (fig. 6). Frequently the intimal changes were limited to the portal vein only and apparently had not yet extended to its main tributaries. In the mesenteric vein muscular hypertrophy was fairly common when the portal vein showed this change.

The *inferior vena cava* was frequently the site of similar or more severe changes. Longitudinal muscle fibres were found beneath the unevenly thickened intima. At the thinner places there was mostly longitudinal muscle (fig. 8), while in the thicker patches the muscle fibres were replaced to some extent by fibrous tissue—replacement fibrosis (fig. 9). From these observations I have formed the impression that the former represents the early stage and the latter the late stage of the same process. It is conceivable that at a still later stage all the muscle fibres may be replaced by fibrous tissue and the change may be called “phlebosclerosis.” The circular fibres in the media remain unaltered. As a rule there is no appreciable muscular hypertrophy in the media but the longitudinal muscle in the adventitia is greatly hypertrophied.

The frequency of intimal changes in the *portal vein*. Among the 28 normal controls not a single instance of intimal thickening was observed, while in the 26 cases of the group with increased

ADAPTATION TO INCREASED INTRAVENOUS PRESSURE



FIG. 4 — Portal vein in longitudinal section, showing slight thickening of intima and marked muscular hypertrophy in the media. The strands of muscle fibres are thicker than normal. The adventitia is not included. H and E.

FIG. 5 — Portal vein, showing thickened intima and longitudinal muscle fibres beneath the endothelium. The section was cut transversely and somewhat obliquely. H and E.



FIG. 6 — Splenic vein in transverse section, showing the same changes as in fig. 5.



venous pressure, hereafter referred to as the pathological group, 20, or 77 per cent., showed intimal changes. As the difference in incidence of these changes in the two groups (77 per cent.) is almost six times its standard error (13 per cent.) it must be considered highly significant, *i.e.* very unlikely to arise from chance.

The coexisting intimal changes in other vessels. In the cardiac cases intimal thickening and muscular hypertrophy usually involve both portal vein and inferior vena cava but are found infrequently in the mesenteric and splenic veins. In early cirrhosis both portal vein and inferior vena cava are frequently affected, the changes in the latter being as a rule the more marked. Different stages are sometimes observed, varying from early intimal thickening and development of longitudinal muscle fibres beneath the intima to the late stage of replacement fibrosis.

The changes in the portal vein seem to be closely related to those in the inferior vena cava but much less frequently to those in its chief tributaries, the splenic and mesenteric veins. A χ^2 test has been applied to the data and shows significant differences between the frequency of occurrence of intimal changes in the portal, mesenteric and splenic veins, but no difference between the portal vein and inferior vena cava.

The question arises as to why the inferior vena cava should be involved in cirrhotic cases and show, in fact, even more marked changes than those of the portal vessels. The explanation may be that with the progress of portal obstruction as the result of advancing cirrhosis and the development of a collateral circulation more blood passes into the inferior vena cava, thus causing an increase of pressure in that vessel. According to Thoma (1883), with a given pressure the tension on the wall is directly proportional to the diameter of the tube and since the inferior vena cava has a greater diameter than the portal vein, the changes in its wall will be more marked.

In a few instances (6 out of 22) the intima of the inferior vena cava was affected despite the absence of cardiac or hepatic disease. This compares with 17 out of 24 in the pathological group. It is natural to ask whether these two groups belong to the same population, the difference in the frequency of their occurrence being ascribed to random sampling. A χ^2 test was used to investigate this possibility (table I).

The test shows that these two groups do not belong to the same population, and are not the result of the same factors.

Medial muscular hypertrophy. Qualitative change. Medial muscular hypertrophy is often associated with the intimal changes in the portal system and appears to be more frequent in the main trunk than in the tributaries. A χ^2 test has been used to test this point and shows that for the comparison between portal vein and

mesenteric vein χ^2 is low and P falls between 0.7 and 0.6 so that there is no significant difference in the proportion of cases with muscular hypertrophy in these two groups of vessels. On the other hand, the comparison between the portal and splenic vein gives a high χ^2 , whilst P falls between 0.01 and 0.001. Hence

TABLE I

*Frequency of intimal changes in the inferior vena cava
in the pathological and normal groups*

Inferior vena cava	Pathological group	Normal group	Total
Showing intimal changes . . .	17	6	23
Showing no intimal changes . . .	7	16	23
Total	24	22	46

$$\chi^2 = 8.76; n = 1; P \text{ between } 0.01 \text{ and } 0.001.$$

there appears to be a significant difference in the proportion of cases with muscular hypertrophy in these two groups of vessels. It has already been shown that intimal changes are more common in the portal vein than in the splenic and mesenteric veins so that it appears that intimal and medial changes are more frequent in the portal vein than in the splenic vein, whereas in the mesenteric vein intimal changes are less frequent, though muscular changes occur as frequently as in the portal vein. These comparisons suggest that, at any rate in the tributaries, muscular hypertrophy precedes intimal changes. This idea is borne out by histological study, for although most vessels showing intimal changes also present medial hypertrophy, on the other hand some vessels show muscular hypertrophy without intimal change.

These changes take place first in the portal vein, then extend to the mesenteric and lastly to the splenic vein. The relative infrequency of changes in the splenic vein is striking and hard to explain. Several explanations may be suggested. (1) It may be due to the fact that most of the cases studied are early cases which have not advanced far enough to affect all the tributaries of the portal vein. In one case where the cirrhotic change in the liver was far advanced both splenic and mesenteric veins were markedly involved. (2) It may be due to the smaller calibre and different structure of the wall of the splenic vein. Normally the splenic vein has a relatively greater proportion of muscular media, which may be able to deal with a pressure higher than that which other vessels can deal with. At present I am not in a position to draw definite conclusions.

ADAPTATION TO INCREASED INTRAVENOUS PRESSURE

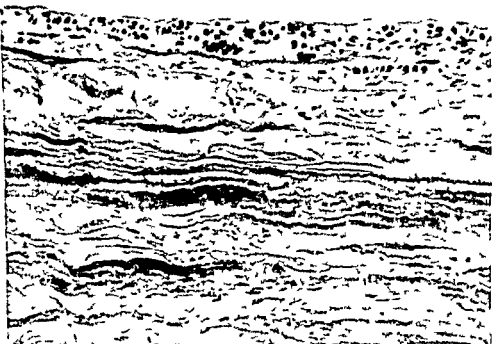


FIG 7—Normal inferior vena cava in longitudinal section, showing very thin intima and thin strands of muscle fibres in the media H and E

FIG 8—Inferior vena cava in longitudinal section, showing thickening of intima with development of longitudinal muscle fibres. Early stage of intimal hypertrophy H and E

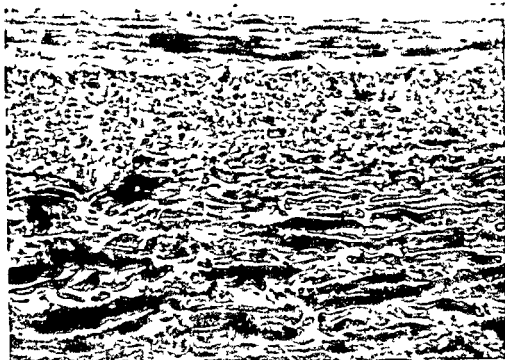
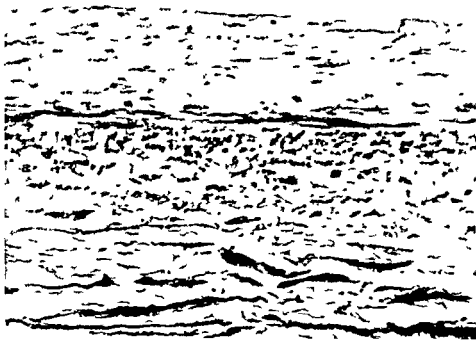


FIG 9—Inferior vena cava in longitudinal section, showing marked thickening of intima with increase of fibrous tissue and decrease of muscle fibres. Taken from the same specimen as fig 8. The changes are more advanced and indicate early replacement fibrosis H and E



In the inferior vena cava there is no muscular hypertrophy in the media.

Quantitative change. Fisher's *t* test has been used for the comparison of the mean widths of the corresponding coats of the portal, mesenteric and splenic veins and the results are recorded in table II.

TABLE II

Comparison of mean width (in μ) of coats of portal, mesenteric and splenic veins in the pathological group and the normal group

	Portal vein			Mesenteric vein			Splenic vein		
	Media (μ)	Adventitia (μ)	Total width (μ)	Media (μ)	Adventitia (μ)	Total width (μ)	Media (μ)	Adventitia (μ)	Total width (μ)
Pathological group	92.12	361.23	453.35	97.45	252.33	349.78	78.66	169.57	248.23
Normal control	73.68	326.43	400.11	82.63	255.05	337.68	76.50	148.24	224.74
Increase	18.44	34.80	53.24	14.82	-2.72	11.90	2.16	21.33	23.49
<i>t</i>	2.596	0.875	1.660	1.837	0.082	0.505	0.817	1.385	0.726
<i>n</i>	52	52	52	35	35	35	50	50	50
P between	0.02 and 0.01	0.4 and 0.3	0.2 and 0.1	0.1 and 0.05	1 and 0.9	0.7 and 0.6	0.5 and 0.4	0.2 and 0.1	0.5 and 0.4

From this table it is evident that in the portal vein in the pathological group the width of the media is significantly increased but the increase in the width of the adventitia and in the total width is not significant. The *t* value of the difference in means of the media of the mesenteric vein is 1.837; *P* from Fisher's table is between 0.1 and 0.05. This is below the usually accepted level of significance. In view of the fact that histological evidence of the muscular hypertrophy is present in most of these cases I am inclined to interpret this borderline value as significant. In the other measurements of the mesenteric and splenic veins, *i.e.* the adventitia and total width, the differences are not significant. It has been established therefore that a true hypertrophy of the medial muscle exists in the portal vein; it may possibly be present in the mesenteric vein also, but the data are not sufficient to provide conclusive evidence of this.

Quantitative changes in the inferior vena cava. Fisher's *t* test has also been applied to the measurements of the inferior vena cava. Since within the normal group of controls (22 cases) 6 show intimal changes similar to those in the pathological group, the question arose whether these 6 should be included as normal controls or not. Their means were compared with those of the

remaining 16 cases of the normal control group by the *t* test. Because there is a significant difference in the means of the adventitia and total width of the two groups these 6 cases were excluded from the class of controls. Comparisons were made between the remaining 16 normal controls and the 23 cases in the pathological group (table III).

TABLE III

Comparison of the width (in μ) of the coats of the inferior vena cava in 16 normal controls and 23 pathological cases

	Media	Adventitia	Total width
23 pathological cases . . .	82.74	550.09	632.83
16 normal controls . . .	60.94	412.94	473.88
Difference	21.80	138.15	158.95
<i>t</i>	1.526	2.777	3.119
<i>n</i>	37	37	37
<i>P</i> between	0.2 and 0.1	0.01 and 0.001	0.01 and 0.001

There is no significant difference between the mean widths of the media of the two groups, but the differences in the widths of adventitia and total width are highly significant.

Summary

In cases of cardiac failure and cirrhosis of the liver, under conditions where the intravenous pressure is increased, there are usually changes in the portal vein and inferior vena cava, whilst the mesenteric and splenic veins are less frequently affected. The changes include (1) intimal thickening with development of longitudinal muscle beneath it and (2) muscular hypertrophy. In the portal vessels the hypertrophy is located in the circular muscle of the media; in the inferior vena cava it affects the longitudinal muscle of the adventitia. It seems likely that the muscular hypertrophy is the initial reaction, the intimal changes developing later.

DISCUSSION

Pathogenesis of phlebosclerosis

Although Morgagni (1769) described a case of calcification of the inferior vena cava which came to his notice as early as 1707 and Baillie (1793) mentions ossification at the bifurcation of the inferior vena cava, Lobstein (1833) is usually given the credit of being the first to recognise phlebosclerosis. He states that veins are often hardened and thickened, especially in the inferior extremities of old people. He suggests that these changes are the result of hypertrophy or of inflammation. Later writers (Fischer, 1900, p. 509; Schilling, 1926) sometimes misquote him as including calcification in his definition. von Rokitsansky (1844) regards phlebosclerosis as hypertrophy of the whole vein wall: there is intimal thickening and increase in width of other coats, with sclerosis. As the cause, he suggests on the one hand mechanical strain resulting from prolonged retardation of the venous stream and chronic stasis, on the other a chronic inflammatory process. In

the opinion of Sack (1888) phlebosclerosis is analogous to arteriosclerosis. The condition is observed more frequently where the normal venous pressure is high. Sack found that in the veins of the upper extremities and neck pathological changes were infrequent, but fibrous thickening of the intima of the veins of the lower extremities, where the venous pressure is usually higher, was quite common. The lesions were described as multiple and circumscribed, but extensive in distribution. The only retrogressive change was slight hyaline degeneration, calcification and fatty degeneration were not seen. He thought that abnormal strain on the vein wall played an important role in the production of the changes but under the influence of his teacher Thoma he assigned first place to some nutritional disturbance which weakens the wall. The localised fibrous thickening of the intima which he called endophlebitis was considered to be a special form of the same condition, caused by local venous stasis.

Fischer (1900) supported Koster's view that phlebosclerosis is caused by inflammation which weakens the vein wall. In four cases of venous sclerosis with intimal thickening he demonstrated inflammatory changes and he regarded abnormal strain as an accessory factor only in the causation of the disease.

According to Kaya (1907) degeneration of the vein wall is rare. Fatty degeneration and calcification are never seen. He described thickening of the intima with development of longitudinal muscle fibres therein and frequently muscular hypertrophy beneath the thickened area. He denied Thoma's, Sack's and Koster's theories because he could not find evidence of primary weakening of the media or inflammatory reaction. The thinning out and fibrosis of the media were considered to be the result of the disease. In his opinion the origin of phlebosclerosis must be sought in a purely mechanical factor, i.e. abnormal strain on the vein wall due to increased pressure.

Faber (1912) occasionally observed calcification in veins from both old and young subjects at places where the mechanical strain was great, but he did not ascribe much importance to this factor. He thought previous weakening of the vein wall was more important. Inflammatory and toxic factors were emphasised. Cramer (1921) and Schilling (1926) described a circumscribed thickened patch with occasional calcification in the posterior wall of the inferior vena cava immediately above the site of bifurcation. They explained the predilection for this site by the fact that the vein is fixed there and is subjected to the force exerted by the converging iliac streams. Apparently, they supported the mechanical view.

Simmonds (1912) reported 7 cases of phlebosclerosis in the portal vein associated with calcification and hyaline change in the intima. He distinguished primary and secondary portal sclerosis, the former almost always caused by syphilis, the latter due to circulatory disturbances, as in cirrhosis of the liver. McIndoe (1928) demonstrated sclerosis of the portal system and hepatic veins in portal cirrhosis. Moschowitz (1932) observed phlebotic changes in the hepatic vein in cases of chronic venous stasis. McMichael (1934) found that the splenic and portal veins were frequently affected by endophlebitis in hepatolienal fibrosis, the histological appearances suggested that it might be caused, at least in part, by increased pressure in the portal venous system. Gross (1937) found similar changes in the hepatic veins and inferior vena cava in cases of cirrhosis of the liver and congestive heart failure. Recently, Gross and Handler (1939) reported identical changes in the superior vena cava in cases of congestive cardiac failure. Weeber (1934) recorded hypertrophic changes with thickening of the intima in the extra hepatic portal radicles, such as the omental veins, in cases of

cirrhosis of the liver and cardiac failure. According to the last five authors, increase of intravenous pressure is the sole cause of the changes in the vein walls.

From the above summary it is evident that there are two factors concerned in the production of phleboscrosis: (1) weakening of the vein wall caused by nutritional disturbances, degeneration of the media or inflammatory changes and (2) increase of venous pressure. Opinion is still divided as to which is the more important and which occurs first. Actually, there seems to be no discrepancy: either can occur first and they are of equal importance. For the production of the lesion an abnormal strain on the vein wall is necessary. If the vein wall is weakened through any cause, a normal venous pressure may prove sufficient to induce intimal changes. On the other hand if the vein wall is normal, prolonged increase in pressure will still cause changes. In the veins studied in this investigation there were no signs of inflammation, therefore venous hypertension must have been the primary cause.

Furthermore, there are numerous clinical and experimental observations to support this view. Clinically it is well known that hypertrophic changes occur in the vein wall in cases of arterio-venous aneurysm or anastomosis where the arterial pressure is lowered but the local venous pressure elevated.

Hermannes (1923-24) described hypertrophic changes in both intima and media in cases of arterio-venous aneurysm. Reid (1931) observed hypertrophic changes in the jugular vein of a woman 34 years old, who had an arterio-venous anastomosis between the carotid artery and jugular vein of 32 years' duration. There were no vascular lesions elsewhere. Experimentally Carrel (1907) demonstrated that veins adapt themselves very quickly to changes of pressure. When a circulation of arterial blood is established through a vein by uni-terminal anastomosis, the vein wall rapidly becomes thickened. The degree of thickening is dependent upon the increase of blood pressure. If the increased pressure is lower than in the anastomosed artery, the vein wall becomes considerably thicker and its lumen dilates. It seems that the reaction stops when the thickening has reached a certain degree. On the contrary, when the pressure is greater than in the artery, the venous thickening is very marked. In this case, however, the reaction does not stop and may ultimately lead to complete obliteration of the lumen. When a venous segment is inserted into an artery by bi-terminal anastomosis, it rapidly undergoes profound modification. There is no dilatation of the lumen of the vein, but the vein wall reacts against the arterial pressure by thickening. Five months after the operation, a venous segment transplanted on the carotid artery exhibits the appearance of an arterial segment, pulsates as an artery, and its circulation is very active.

These examples are of special significance because every factor except increased pressure transmitted directly from the artery can be excluded. Wohlwill and Holm (1927) obtained similar changes in the portal vein by producing a reversed Eck fistula, *i.e.* by anastomosing the inferior vena cava to the portal vein. In this

case the increased portal pressure was due to increased in-flow. It seems certain, therefore, that whatever the primary cause of venous hypertension the reaction is the same, resulting in phlebosclerosis.

Histology

As revealed by the present investigation, the special features are (1) thickening of intima with development of longitudinal muscle fibres beneath the intima and replacement fibrosis in the later stages and (2) hypertrophy in the muscular coat.

Intimal changes have been described (Epstein, 1887; Sack, 1888; Kaya, 1907; Gross, 1937; and others) in connection with phlebosclerosis, in arterio-venous anastomosis and arterio-venous aneurysms (Wiedmann, 1893; Hermannes, 1923-24; Reid, 1931) and also in experimental arterio-venous anastomosis (Carrel, 1907; Borst and Enderlen, 1909; Fischer and Schmieden, 1909; Stich and Zoepfritz, 1909).

The muscular hypertrophy is less often mentioned. Kaya described medial muscular hypertrophy beneath the thickened intima, while Gross has seen it in the media of the hepatic veins and *venae cavae*. In descriptions of arterio-venous anastomosis and aneurysms the medial muscular hypertrophy has been a constant feature (Bramann, 1886; Wiedmann, 1893; Carrel, 1907; Fischer and Schmieden, 1909).

The present investigation reveals a selective localisation of muscular hypertrophy: in the portal vein it affects the circular muscle of the media, in the inferior vena cava the longitudinal muscle of the adventitia. I am grateful to Dr K. J. Franklin for the following explanation of this difference. The inferior vena cava is fixed at both ends so that hypertrophy of the longitudinal muscle will be sufficient to deal with distension caused by increased internal pressure. The portal vein on the other hand is only fixed at its upper end, the lower being free to move about. Hypertrophy of longitudinal muscle would add to the distension, since its contraction shortens the vessel and increases the width of the lumen. Hypertrophy of the circular muscle in the media is thus called for.

No previous suggestion has been made as to which of the two changes, intimal thickening and muscular hypertrophy, occurs first. In my opinion muscular hypertrophy precedes intimal change, for in most cases showing the latter, muscle hypertrophy was present, whereas cases with muscular hypertrophy sometimes showed no intimal change. The hypertrophic muscle gradually fails and undergoes degeneration and absorption, becoming replaced by fibrous tissue (Kaya, 1907; Gross, 1937). The result is that the thickened muscular coat becomes fibrosed to a varying degree. There is also replacement fibrosis of the longitudinal muscle in the thickened intima. This late stage of the disease conforms to the ordinary description of phlebosclerosis. Histologically this

series of changes is analogous to those seen in the arterial system under the influence of prolonged elevation of blood pressure (Thoma, 1911, 1920 ; Turnbull, 1914-15 ; Muir, 1936).

Histogenesis of the intimal longitudinal muscle

Longitudinal muscle fibres are described as being present in the intima of the iliac, femoral and saphenous veins (Maximow and Bloom, 1938) and dorsal vein of penis (Bremer, 1936), and at the bifurcation of veins (Benninghoff, 1930). It would seem that normally they occur in small numbers at places where the venous pressure is obviously high. I have never seen well defined longitudinal muscle fibres in the intima of a normal portal vein or inferior vena cava, although not infrequently I have observed beneath the endothelium a few oval and elongated nuclei which might be the nuclei of connective tissue cells or of precursors of smooth muscle cells.

Under pathological conditions, where the intravenous pressure is elevated, the development of longitudinal muscle fibres in the thickened intima is quite common. They were seen, for example, in most cases of the pathological group in the present investigation. Previous workers on phlebosclerosis, arterio-venous aneurysm and experimental arterio-venous anastomosis have noticed their occurrence in the thickened intima. It is very likely that the *anlage* of these muscle fibres is present in the vein wall, but for its development an elevated venous pressure is essential.

For a clear understanding of the nature and behaviour of the intimal longitudinal muscle fibres in veins it seems desirable to review their analogous structure in the arterial system. In the aorta there appears first a layer of longitudinally directed muscle and elastic fibres, the musculo-elastic layer of Thoma (1883), and later on the hyperplastic layer of Jores (1903), consisting of tiers of circularly directed elastic and muscle fibres. A musculo-elastic layer is present in the thoracic aorta at birth and appears in the abdominal aorta within two or three months after birth. The hyperplastic layer is usually well developed at the age of ten years ; it appears first in the thoracic aorta, sometimes as early as the third year. In other large elastic arteries similar musculo-elastic and hyperplastic layers are developed, but in such arteries the development of these layers exhibits more focal variation than in the aorta. In the muscular arteries the intima develops as in the elastic arteries, but under normal conditions it is much more focal and of less magnitude. Hypertrophy of the musculo-elastic layer in the small arteries accompanies hypertrophy of the muscular coat in arterial hypertension. Intimal hypertrophy is also found in focal high blood pressure, *e.g.* in the pulmonary artery in cases

of mitral stenosis when resistance is increased in the pulmonary circulation. Hypertrophy of the intima, according to Turnbull, may occur independently of high pressure but is invariably associated with histological evidence of degeneration of the media in the arteries. This seems to suggest that when the vessel wall is weakened a normal blood pressure may become too high for the vessel and induce changes in the intima. All these facts about arteries can be paralleled in the venous system, and it is no exaggeration to compare the thickening of the intima and the associated development of longitudinal muscle fibres in veins with the behaviour of the musculo-elastic layer of the arterial system in hypertension.

Origin of the intimal longitudinal muscle

There is no doubt about the presence of smooth muscle fibres in the thickened intima associated with increased intravenous pressure, but under normal conditions the intimal muscle fibres are scanty and far from obvious. Kaya suggested that they take their origin from the circular muscle of the media. Since they are longitudinal in direction and usually separated from the media by the internal elastic lamina it is hard to agree with this view. Borst and Enderlen believed that they are derived from the cells of the intimal "polster" which is developed from the lining endothelial cells. Some of the cells of this "polster" differentiate into connective tissue, others into muscle cells. When vein segments were inserted into arteries in dogs, it was found that the intimal connective cells decreased whilst the muscle cells increased. Fischer and Schmieden found in the thickened intima of an arterialised vein a thick layer of large spindle-shaped cells; most of these appeared to be connective tissue cells, whereas others closely resembled muscle cells. In their opinion it was difficult to decide the origin of these muscle cells. Hermannes gave further evidence in support of the view of Borst and Enderlen. He described transitional forms between the undifferentiated polygonal cells of connective tissue type and the well developed spindle-shaped muscle cells. The latter were located near the media, i.e. in the outer part of the intima. He agreed with Borst and Enderlen that the intima is the product of endothelial cells and that development extends from the lumen of the vessel towards the media. The proliferated intimal cells differentiate into connective tissue and muscle cells. He suggested that the pulsatory changes of pressure may determine the direction of the developing muscle fibres. Maximow and Bloom described partly differentiated muscle cells in veins of small calibre. These observations suggest either the presence within the intima of veins of a mechanism by which connective tissue can be converted into smooth muscle or of the

persistence of primitive undifferentiated muscle cells in that situation. From the facts mentioned before there is ample reason to believe that the development of the intima and its muscle depend upon the functional stimulus caused by increased intravascular pressure.

It is my belief that the hypertrophy of the intima and muscle coat of the vein wall is the early stage of the so-called phlebosclerosis. It is a reaction of the vein wall to increased mechanical strain, being the result of increased venous pressure. Moreover it is identical in pathogenesis and histology with the adaptive hypertrophic changes found in the arterial system in hypertension (Turnbull, 1914-15; Muir, 1936). I would suggest therefore that such a change in veins is adaptive in nature.

Adaptability of the portal vein

Age exerts no important influence on the adaptability of the portal vessels. Neither does it appear that the portal vein grows old. According to Mann and his associates (Mann and Magath, 1922; Mann, 1932) the capacity of the liver for regeneration depends upon a good blood supply, especially of portal blood. Professor Cameron tells me that the liver in old rats can regenerate after partial hepatectomy as effectively as in young ones. Col. Hamerton, pathologist to the Zoological Society, London, has informed us that he has frequently noticed evidence of recent liver regeneration in old monkeys. The present findings are in complete agreement with these data.

SUMMARY AND CONCLUSIONS

1. The normal and pathological histology of the portal, mesenteric and splenic veins and the inferior vena cava have been studied and the thickness of their coats measured.

2. In cardiac and cirrhotic diseases—conditions in which the intrahepatic pressure is presumably elevated—changes are usually found in these vessels. These include (i) thickening of the intima with development of longitudinal muscle fibres beneath it and (ii) muscular hypertrophy in the vein wall. In the portal vessels the hypertrophy affects the circular muscle of the media, while in the inferior vena cava it is located in the longitudinal muscle of the adventitia.

3. The muscular hypertrophy is believed to develop first, the intimal changes following.

4. Such changes are considered to be an adaptive mechanism in response to increased intravenous pressure similar to the changes in arteries in hypertension.

5. The association of changes in the portal vein and inferior vena cava is very close, but those in the latter are usually the more

marked The mesenteric vein is a direct continuation of the portal vein and frequently shows similar changes but of milder degree The splenic vein is less frequently involved

6 The adaptability of the portal vessels is not influenced by age Veins of elderly subjects can hypertrophy as much as those of young subjects

7 Atheromatous change has not been seen in the veins examined It must therefore be regarded as a separate entity and of rare occurrence

I wish to thank Professor G R Cameron for the suggestion of this study and his constant interest in the work and help in preparing the manuscript My thanks are also due to Dr K J Franklin who has generously supplied me with much information concerning the physiology of the veins and to Dr Matthew Young and Mr C T Hsu for help with the statistical analyses

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CULTURAL METHODS AS AN AID IN TYPE DIFFERENTIATION OF GROUP A HÆMO- LYTIC STREPTOCOCCI

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THE immediate adoption by bacteriologists of the precipitin methods introduced by Lancefield, which established a rational classification coinciding with the natural habitats and pathogenic potentialities of the strains, was the best indication of the deficiencies of former classifications of streptococci by means of fermentation tests. As Topley and Wilson remark in the first (1929) edition of their *Principles of bacteriology and immunity* (p. 360) the abandonment of these tests (*i.e.* fermentation reactions) "does not, however, involve the view that they yield no information of value. Although the conclusions of Gordon, and of Andrews and Horder, have been criticized by subsequent observers, there is a considerable body of evidence that the differences noted by them do, in fact, correspond in a broad and general way to differences in habitat." No one who rereads, in the light of recent advances, the studies of Gordon, Andrews and Horder, Holman and others, can fail to note how closely they approached solution of the problem of correlation of physiological characters with pathogenic powers. On examining the details of the sources of the strains classified by Holman, it is possible to identify with some plausibility the groups of many, and even in some cases, the serological type.

Stimulated by the suggestions of Sherman (1937), we have examined the fermentation reactions of a collection of streptococci which had already been classified by serological methods. We find that the various type strains (Griffith) within group A (Lancefield) have characteristic physiological reactions on which can be based a classification similar to that obtained by slide agglutination methods, though at present less complete.

The tests employed to classify the types within group A are (a) the colonial form in a serum peptone agar medium, (b) ability to produce opalescence in serum broth and (c) ability to attack mannitol, starch and cellobiose. The first two tests constitute the presumptive test of Ward and Rudd (1938) for identification of group A streptococci. Mannitol has been used since the work of Gordon (1905). The possibilities of starch were noted by Andrews (1930) and attention was recently redirected to his work by Sherman (1937). Cellobiose was tested after reading an excellent review by Jensen (1910), who stated that H. Holth, working in

his laboratory, had tested 150 strains of streptococci on all procurable carbohydrates and polyvalent alcohols, as well as many glucosides, and had found lactose, trehalose, cellobiose and gentiobiose to be of differential value.

METHODS AND MATERIAL

Numbers, sources and types of strains

The type strains of group A were generously supplied by Dr F. Griffith. The local strains, both those falling into the Griffith series and those belonging to other types identified locally, were selected from over 1100 group A strains collected at these Laboratories from various Australian sources during the past year. A large number have been tested by the methods described but the results recorded were obtained with a selected series. We attempted to include 15-20 strains of each serological type from various sources, but in some cases had to be content with smaller numbers. In only one type (17) did the characteristics of the English and local strains differ. The numbers and origins of the local strains are shown in table I. Type "Wade",

TABLE I

Numbers and sources of locally isolated strains of group A streptococci

Type	Scarlet fever; cases and contacts	Tonsillitis and sore throat	Abscesses and pus	Puerperal	Excised tonsils	Healthy throats	Erysipelas	Fæces	Total
1	1	0	1	2	0	0	1	0	5
2	20	0	1	0	3	0	0	0	24
4	4	2	1	2	9	2	0	0	20
5	0	0	1	4	0	0	0	0	5
6	3	4	6	0	8	0	0	0	21
9	1	1	0	0	1	2	0	0	5
11	9	2	1	3	3	1	0	0	19
12	1	3	1	2	4	0	0	1	12
17	9	1	3	0	5	0	0	0	18
19	4	0	0	0	0	0	0	0	4
22	1	0	0	5	5	7	0	0	18
27	1	4	3	2	4	1	0	0	15
28	0	0	0	0	0	2	0	0	2
"Wade"	18	0	0	0	2	0	0	0	20
"Stewart"	0	1	3	1	6	7	0	0	18
"Woodbury"	2	0	0	6	0	1	0	0	9
"Alli"	5	2	3	1	8	1	0	0	20
"Coleman"	1	0	0	0	4	0	0	0	5
"Hale"	1	0	0	1	2	2	0	0	6
Totals . .	81	20	24	29	64	26	1	1	246

originally identified by H. K. Ward in Sydney, was isolated from 5-10 per cent. of the scarlet fever cases in Melbourne and from 90 per cent. of the Adelaide cases during the past year. Type "Stewart" was very rarely found in association with scarlet fever, but was frequently isolated from the healthy throat, from patients with sore throat or tonsillitis, and from surgical infections (cellulitis, abscesses, empyemata, otitis, etc.). Types "Wade" and "Stewart" are serologically closely linked to each other and to type 9 (Griffith). There are 143 "Wade" and 49 "Stewart" strains in this

collection Type "Woodbury" was isolated from a case of puerperal fever by Miss Hildred Butler in December 1937. The type serum we prepared from this strain specifically agglutinated 5 other strains isolated from patients with puerperal fever in the same ward in the ensuing five weeks, as well as a strain which had been isolated from a post-abortal case in the same hospital a month previously. The other sources from which it was recovered are shown in table I, as are the sources and numbers of types "Hale", "Coleman" and "Alli".

Differential tests

According to Ward and Rudd (1938) hemolytic streptococci belonging to group A produce either feathery colonies in serum peptone agar, with or without opalescence in serum broth, or compact colonies with opalescence, those belonging to other groups form compact colonies without opalescence. There are rare exceptions to the general rule (Ward and Rudd, 1938, 1939). No strains from human sources in our collection which belong to groups other than A have produced opalescence in serum broth, though three canine strains do so. Occasionally group A strains, usually from pus, may give compact colonies and produce opalescence only after a delay of 2-5 days. Only 8 group A strains have failed to conform to the criteria of the presumptive test. Four are untyped and four belong to type 'Coleman'.

The carbohydrates of value for type differentiation are starch, mannitol and cellobiose, the following substances were found valueless for this purpose: lactose, raffinose, salicin, xylose, arabinose, inulin, amygdalin, rhamnose, dulcitol, dextrin, glycogen and aesculin, the reactions being common to all group A strains. Fermentation of glycerol and ability to grow on 10 per cent and 40 per cent blood bile agar varied with different strains of the same serological type. Production of acid and clot in milk seemed promising, but, if the streptococci were inoculated into pure milk, growth was irregular, if the milk was diluted with tryptic broth or aseptic fluid all strains produced acid and clot. These irregularities may be due to the combination of several factors in a single test, acid production from lactose, changes in the redox potential and specific enzymatic clotting.

Media

Serum peptone agar Dissolve 0.25 g shredded agar, 1.0 g neopeptone, 0.85 g NaCl in 100 c.c. of distilled water, adjust pH to 7.6, filter through paper and sterilise in autoclave in flask A. The consistency of the contents of this flask, when cold, should be that of porridge and not of a solid gel and the agar content may have to be altered to attain the desired consistency. Into flask B measure 25 c.c. of tryptic broth, 50 c.c. of 0.85 per cent NaCl solution and 25 c.c. of normal horse serum (not more than 8 weeks old) which has been heated at 56° C for 30 minutes. Melt the contents of flask A, cool to 50-55° C and add to flask B, warmed to the same temperature. Mix and tube in 3.0 c.c. amounts in sterile 3 × $\frac{1}{2}$ inch test tubes. Store in refrigerator and in any case cool in refrigerator before use.

Serum broth Mix three parts of horse serum with one part of tryptic digest broth, distribute with a sterile pipette in 3.0 c.c. volumes in tubes and store in the refrigerator.

Mannitol and cellobiose These substances are used in 0.5 per cent concentration in a basic medium (pH 7.1) containing proteose peptone (Difco) 10 g, NaCl 5 g, and water to 1 l, with brom cresol purple as indicator (0.1 per cent of 1.6 per cent solution in 95 per cent ethyl alcohol) and sterilised by Seitz filtration. In the case of cellobiose, 10 per cent asetic fluid was added. This may be unnecessary, but we have not tested sufficient

strains without the addition of ascitic fluid to be positive on this point. The sample of cellobiose was prepared by Dr K. Welch of these laboratories.

Starch is used in a medium containing proteose peptone (Difco) 10 g., NaCl 5 g., soluble starch (B.D.H.) 1 g., Andrade's indicator 10 c.c. and water to 1 l., warmed to dissolve, tubed in 3 c.c. quantities and autoclaved for 15 minutes at 115° C.

Technique

The preparation and inoculation of the serum peptone agar tubes are the only technical processes which present any difficulty. It is essential to obtain a medium of the proper consistency and to distribute the inoculum so that 5-20 isolated colonies are evenly distributed throughout the medium. Using a loop, 1 mm. external diameter, of 28-gauge platinum wire, one loopful of an 18-hour broth culture is inoculated into the serum broth tube (3 c.c.). The loop is removed and flamed and the contents of the serum broth tube thoroughly mixed. One loopful of this dilution is transferred to the serum peptone agar tube. The loop is plunged rapidly to the bottom of the tube and twirled in the medium. The contents of the tube are then thoroughly mixed by rotation and lateral tilting, taking care not to introduce air bubbles. With a little practice and adjustment of the size of the loop, satisfactory results are readily obtained.

The "feathery" colonies are usually spherical, sometimes elongated, semi-transparent masses 3-4 mm. in diameter. The compact colonies are tiny dense masses about 1 mm. in diameter. These appearances are fully described and figured and the possible fallacies of the test discussed by Ward and Rudd (1938, 1939).

RESULTS

The findings are shown in table II. With the aid of these tests, our strains can be divided into 12 out of 32 possible groups (24 if

TABLE II
The differential reactions of group A streptococci

Types of locally isolated strains	Colony in serum peptone agar	Opalescence in serum broth	Fermentation of			Griffith's type strains
			mannitol	starch	cellobiose	
2, 9	F	+	—	+	+	2, 9, 28
...	F	—	—	+	—	10
1	F	—	—	+	+	1
6	F	—	+	—	(—)	6, 17
...	F	—	—	—	—	15
...	F	+	—	—	+	13
5, 12,* 17, 19, 27*	F	—	—	—	+	3, 5, 11, 14, 18, 19, 24, 26, 29, 30
4, 28, "Wade"	C	+	—	+	+	4
"Hale"	C	+	+	—	(—)	23
11, 12, 27, "Stewart,"	C	+	—	—	+	12, 25, 27
"Alli"						
22	C	+	—	—	—	8, 22
"Woodbury"	C	+	—	+	(—)	...
"Coleman"	C	—	—	+	+	...

* One strain only gave these reactions.

F = feathery colonies; C = compact colonies.

(—) = may ferment cellobiose from the fifth day onwards.

those including compact colony without opalescence are excluded). Since there are more than 30 serological types within group A, complete type differentiation cannot be effected by this means. Six of the groups, however, contain only a single serological type, so that six of the types encountered in Melbourne can be presumptively identified by these tests alone, namely types 1, 6, 22, "Hale", "Coleman" and "Woodbury".

*Comparison of the English and local type strains
in the differential tests*

The Griffith type strains give the same reactions as local isolations, except types 11, 17 and 28. The English type strains 11 and 28 produce feathery colonies in serum peptone agar; type 11 does not produce opalescence in serum broth, type 28 does. The Australian strains of these types give compact colonies with opalescence. This variation in these two tests in strains of the same serological type was noted in the original communication of Ward and Rudd (1938). Such discrepancies do not seriously impair the utility of the tests in type differentiation, since locally isolated strains are consistent in their reactions, at any rate in Melbourne.

The English type 17 strain attacks mannitol and is a slow fermenter of cellobiose; the Australian type 17 strains have no action on mannitol but rapidly ferment cellobiose. As types 15, 17 and 23 are hard to distinguish serologically (Griffith, 1934; Pauli and Coburn, 1937; Coburn and O'Connell, 1939; Neisser, 1939), this may not be a genuine discrepancy, but the identification of the Australian strains as type 17 may be at fault. We have no certain Australian representatives of types 15 and 23, though types "Hale" and 23 are closely related serologically and are probably identical. If findings with these types can be confirmed on a larger series, it should be easy to distinguish type 15 from types 17 and 23 by the actions on mannitol and cellobiose.

Such locally isolated strains as attack mannitol and starch almost always give clear-cut results on overnight incubation. Delayed starch fermentation is characteristic of certain strains of other serological groups. The Griffith type strains may not show the typical reaction until the second day of incubation or later, and inconsistent results are occasionally obtained when the inoculum is taken directly from an old blood agar culture. Two or three rapid passages in a favourable liquid medium are advisable.

With cellobiose also, local isolations give a definite reaction on overnight incubation. Type 6 strains may or may not attack it after several days. Of 20 strains tested, 5 were positive between the 10th and 14th days. Type "Woodbury" strains are characteristically slow fermenters of cellobiose; all strains tested were positive between the 5th and 7th days. Type 22 strains were

1. The first part of the document is a list of names and their corresponding dates. The names are: "John", "Mary", "James", "Elizabeth", "Thomas", "Sarah", "Michael", "David", "William", "Richard", "Robert", "Henry", "George", "Charles", "Edward", "John", "Mary", "James", "Elizabeth", "Thomas", "Sarah", "Michael", "David", "William", "Richard", "Robert", "Henry", "George", "Charles", "Edward". 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those including compact colony without opalescence are excluded). Since there are more than 30 serological types within group A, complete type differentiation cannot be effected by this means. Six of the groups, however, contain only a single serological type, so that six of the types encountered in Melbourne can be presumptively identified by these tests alone, namely types 1, 6, 22, "Hale", "Coleman" and "Woodbury".

*Comparison of the English and local type strains
in the differential tests*

The Griffith type strains give the same reactions as local isolations, except types 11, 17 and 28. The English type strains 11 and 28 produce feathery colonies in serum peptone agar; type 11 does not produce opalescence in serum broth, type 28 does. The Australian strains of these types give compact colonies with opalescence. This variation in these two tests in strains of the same serological type was noted in the original communication of Ward and Rudd (1938). Such discrepancies do not seriously impair the utility of the tests in type differentiation, since locally isolated strains are consistent in their reactions, at any rate in Melbourne.

The English type 17 strain attacks mannitol and is a slow fermenter of cellobiose; the Australian type 17 strains have no action on mannitol but rapidly ferment cellobiose. As types 15, 17 and 23 are hard to distinguish serologically (Griffith, 1934; Pauli and Coburn, 1937; Coburn and O'Connell, 1939; Neisser, 1939), this may not be a genuine discrepancy, but the identification of the Australian strains as type 17 may be at fault. We have no certain Australian representatives of types 15 and 23, though types "Hale" and 23 are closely related serologically and are probably identical. If findings with these types can be confirmed on a larger series, it should be easy to distinguish type 15 from types 17 and 23 by the actions on mannitol and cellobiose.

Such locally isolated strains as attack mannitol and starch almost always give clear-cut results on overnight incubation. Delayed starch fermentation is characteristic of certain strains of other serological groups. The Griffith type strains may not show the typical reaction until the second day of incubation or later, and inconsistent results are occasionally obtained when the inoculum is taken directly from an old blood agar culture. Two or three rapid passages in a favourable liquid medium are advisable.

With cellobiose also, local isolations give a definite reaction on overnight incubation. Type 6 strains may or may not attack it after several days. Of 20 strains tested, 5 were positive between the 10th and 14th days. Type "Woodbury" strains are characteristically slow fermenters of cellobiose; all strains tested were positive between the 5th and 7th days. Type 22 strains were

all negative after 14 days. Many of the Griffith type strains were slower, the change not occurring until the second to the fourth day. Serial passage in tryptic digest broth and the addition of ascitic fluid to the cellobiose medium resulted in a shortening of the delay.

Salicin is not included in the differential tests because all locally isolated group A strains and the Griffith type strains produced acid overnight in the basic proteose-peptone medium containing 0.5 per cent. salicin. Several brands of salicin were used. Evans (1937) has separated a group which, by the method she employs, are non-fermenters of salicin, and are designated *Streptococcus scarlatinae* in her classification. Dr Evans kindly sent us five representatives of this group. All of them, tested by our methods, produced acid between the third and fifth days and would be classed by us as slow fermenters of salicin. These strains were all specifically agglutinated by the Griffith type 10 serum.

Cellobiose fermentation in relation to pathogenicity

Among Melbourne isolations, types 6, 22, "Woodbury" and "Hale" are the only strains which either do not attack cellobiose or show a delayed fermentation. Type 6 strains are capsulated. Types 22, "Woodbury" and "Hale" produce compact colonies in serum peptone agar, a characteristic of non-capsulated strains. These non-capsulated non-fermenters of cellobiose have some interesting characters. Of 27 strains belonging to type 22, no less than 23 were isolated from patients with puerperal infections. All the infections were mild and no patient died, although only two cases were recent enough to have had the benefit of sulphanilamide therapy.

There are 9 "Woodbury" strains, 5 of which were derived from similarly mild puerperal infections occurring consecutively within a space of six weeks. The same strain had been isolated from a patient with a post-abortion infection in the same hospital a month previously. Type "Hale" was first isolated from a similar case. None of these strains have been isolated from any serious infections. Outside the puerperal infections, they have been isolated chiefly from excised tonsils and apparently healthy throats. The combination of lack of capsulation and failure to ferment cellobiose may therefore be associated with low pathogenicity. There is an *a priori* presumption, and some evidence, that capsulation in the streptococci is associated with enhanced pathogenicity (White *et al.*, 1939).

DISCUSSION

These methods are not proposed as a substitute for serological classification, for final identification is possible with only a few types. We have however found them a great help in routine typing. A preliminary presumptive determination of group and type limits

the number of sera to be tried in final type identification. It is not entirely safe to proceed to slide agglutination tests without prior determination of the group to which the strain belongs. The use of the presumptive test of Ward and Rudd reduces the number of errors arising from apparently specific agglutination of strains of groups B, C and G by type sera of group A. Further, the use of the fermentation tests directs attention to the existence of antigenic differences which might not otherwise be disclosed, a matter of importance in the present state of the serological classification of the hæmolytic streptococci. Using a type 9 serum which is completely specific in its action on the Griffith type strains, we cannot separate types 9, "Stewart" and "Wade". The failure of some strains agglutinated by this serum to attack starch led us to prepare a serum against one of them. Cross absorption tests established the serological individuality of type "Stewart". Similarly many local strains were agglutinated by our type 10 and type 8 sera, which, judged by their action on the type strains, were specific. Since these local strains gave different reactions in the physiological tests, sera were prepared against them. These sera failed to agglutinate Griffith type 10, the cross with type 8 was readily removed by absorption, and the new types "Alli" and "Coleman" were established. We had the same experience with type 28. We feel, therefore, that workers accustomed to serological typing of streptococci who try the methods described may be inclined to discard them if their results fail to coincide with those obtained by slide agglutination. It is our experience that it is almost invariably the serological identification which has been at fault or incomplete. Other discrepancies have occurred with unstable strains identified only after treatment of the suspensions with trypsin or following growth in broth containing free trypsin. These methods often enable a strain to be identified with apparent success, but we now regard any results so achieved with suspicion and prefer to rely on less drastic methods such as growth at room temperature to obtain stable suspensions. It may be that the strains forming unstable suspensions are variants which have lost certain reactions characteristic of the majority of strains within the type. There is, however, no real evidence to support such an assumption. There is in our collection a residuum of such unidentified unstable strains, less than 5 per cent. of the total; these were isolated mainly from excised tonsils.

In laboratories where type sera or workers experienced in the slide agglutination technique are not available, these tests may be useful. It can often be stated with reasonable certainty whether strains isolated from patients and contacts belong to the same or different types and which of them belong to group A, without final identification of type. The possibilities of finding further differential

tests are not yet exhausted, and as this paper is completed we note the report of Fuller and Maxted (1939) that type 3 strains are characterised by lack of peroxide formation.

Finally, we wish to reiterate that we do not put forward these methods as a substitute for typing by the slide agglutination technique but as subsidiary aids. The results were obtained with sufficiently large numbers of Melbourne strains to establish their constancy, but only single representatives of the English strains were examined. The concordance between the reactions of the local and English strains on starch, mannitol and cellobiose strongly suggests fixed type characters. But it is not unlikely that the findings in serum peptone agar and serum broth may vary with strains of the same type in different localities. If so, a knowledge of such local peculiarities will have to be acquired before full use can be made of these reactions. But even if these two tests are employed only in conjunction and as a presumptive test for group A, or not used at all, a useful preliminary division of group A strains can be made with the three carbohydrates.

SUMMARY

1. The hæmolytic streptococci of group A (Lancefield) can be classified by their differential reactions on starch, mannitol and cellobiose, by the colonial form in a special agar medium and by the production of opalescence in serum broth.

2. The resulting classification is concordant with that obtained by the serological methods of Griffith but is less complete.

3. The advantages and disadvantages of these methods are discussed.

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THE CULTURE *IN VITRO* OF LEUCOCYTES FROM HUMAN BONE MARROW

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(PLATES XIX AND XX)

THE bone marrow was one of the first tissues with which the technique of tissue culture was tried. Curiel and Burrows explanted the marrow of a young cat in 1910 and observed the rapid migration of hæmopoietic cells and the later appearance of fibroblasts. The application of tissue culture technique to mammalian hæmopoietic tissue has been attempted by several workers, notably Foot (1913), Maximow (quoted by Bloom, 1938), Erdmann (1917), van Herwerden (1917-18), Grossmann (1923-24), Rasmussen (1933) and Spadafina (1935). Meier, Posern and Weitzmann (1937) were the first to use human marrow, obtained from a rib during a surgical operation. The results of these workers have been somewhat confusing and there seem to be two chief reasons for this. Firstly, by the technique used, the explanting of fragments of marrow into plasma clots soon results in a growth of fibroblasts which obscures the field of activity. Secondly the cultures were studied either by total fixation and staining and direct examination or by preparing sections, usually after celloidin embedding. With these techniques the various types of primitive blood cells all tend to appear similar and to resemble lymphocytes so that it is hardly surprising that many of the above workers—in particular Maximow—have evolved a theory which gives the lymphocyte wide powers of change into other blood cells.

By using cells obtained from the blood instead of the bone marrow, the first difficulty was avoided, consequently the majority of workers have used blood as their starting point. The extensive literature on this subject has recently been reviewed by Bloom (1938). Blood cells are however less satisfactory than marrow cells, since they seem mostly to be mature cells, mitoses for instance are never seen in normal circumstances. Thus the results have again been disappointing and since the same techniques were used they have chiefly demonstrated the versatility of the "lymphocyte."

In 1937 Osgood and Brownlee described a method using human bone marrow obtained by sternal puncture. It was cultured in a fluid medium in rubber-capped vials; by centrifuging the vials the cells could be sufficiently precipitated for the medium to be changed, and by withdrawing a sample of fluid and centrifuging it cells for smear preparations were obtained and could be studied by routine methods. This technique possesses several advantages. (1) Sternal puncture yields marrow cells free of the supporting fibrous tissue and the method of preparation removes the fat cells; there is a mixture with blood cells, but these are known types and do not obscure the changes in the marrow cells. (2) The fluid medium seems to obviate the exaggeration of the polyblast or macrophage type of cell, which is a feature of plasma clot cultures. (3) The fact that the cells can be studied by a smear technique and not necessarily in histological sections is important. Biopsies on human marrow have shown that the nuclear characteristics distinguishing the various types of cells can be clearly detected only in stained smears. The cytoplasmic characteristics are also more easily viewed in such preparations and can be compared with blood and marrow cells stained by the same methods.

In practice the technique of Osgood and Brownlee proved to have several disadvantages; it was difficult to maintain sterility, and the medium often produced a precipitate which obscured the details of the cultured cells. The following modification of their technique has given satisfactory results.

Materials and apparatus

Bone marrow. This was obtained by sternal puncture. A Salah type of needle was used and about 2 c.c. of the mixed marrow and blood sucked into a 2 c.c. Record syringe; the syringe was kept in alcohol and dried with acetone before use.

Saline. The solution recommended by Parker (1938) was used. It contains NaCl 8.00 g., KCl 0.20 g., CaCl_2 0.20 g., $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.10 g., $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.05 g., NaHCO_3 1.00 g., glucose 1.00 g., water (glass distilled) to 1000 c.c. The pH should be 7.4 to 7.6. It was sterilised by Seitz filtration, put into sterile rubber-capped 100 c.c. Pyrex flasks and stored at room temperature.

Heparin. Puro heparin manufactured by Messrs Roche Ltd. was used. This is supplied in 0.9 per cent. saline, 5 mg. per c.c. For cultures it was diluted to 1 mg. per c.c. with Parker saline, put in sterile capped 10 c.c. bottles and stored in the refrigerator.

Placental serum. This was collected for me by the labour ward staff. They were provided with clean sterile 175 c.c. beaker flasks with plugged mouths, each containing 2.5 mg. of heparin. At the labour the umbilical cord was clamped on the maternal side before it was cut; after cutting, the clamp was removed and the cord allowed to bleed into the flask, no special precautions to maintain sterility being taken. The yield from each case was 30-60 c.c. and about 100-120 c.c. were collected in one flask. As soon as possible the blood was taken to the laboratory and centrifuged; the plasma was pipetted off into 25 c.c. culture bottles which were capped and put

in the refrigerator. After 1 or 2 days the plasma clotted; the fibrin was removed and the serum centrifuged and sterilised by passing through a Seitz filter. It was put into 10 c.c. bottles, which were capped, sealed and stored in the refrigerator. Such serum keeps at least 3 months and should show no precipitate.

Culture bottles. These were centrifuge tubes of 25 c.c. capacity with slightly rounded bottoms to fit a "Bara-Gyro" centrifuge; they were of Pyrex glass and had a flanged neck of 1 cm. diameter which could be closed by a rubber cap. The caps were taken out of boiling distilled water, put on when dry, and painted over with a 5 per cent. solution of histological celloidin in equal parts of absolute alcohol and ether.

Wintrobe tubes. These were Wintrobe's type of flat-bottomed hæmatocrit tube, 11 cm. long, 2.5 mm. bore, and taking 0.7 c.c. fluid.

Syringes. Luer-Lok all-glass syringes (2 c.c. and 4 c.c.) fitted with no. 25 hypodermic needles were used for measuring quantities of saline, serum, and marrow cell suspension. These syringes could be manipulated with one hand and the needle could not come off the nozzle when locked. They were kept in alcohol, washed out in boiling distilled water and finally in saline before use. The needles were dry-sterilised in glass covers.

All the glassware was sterilised dry at 155° C. for at least half-an-hour. Manipulations were carried out under a glass canopy.

Method

Preparation of cultures. Into a corked culture bottle are put 6 c.c. of saline and 1 mg. of heparin; 1.2 c.c. of marrow fluid obtained by sternal puncture are run into this bottle and it is well shaken. The cork is replaced by a rubber cap and the bottle centrifuged at 1500 r.p.m. for 15 minutes. As much fluid as possible is removed with a pipette and its volume measured. The amount by which this volume exceeds 6 c.c. is replaced by fresh saline e.g. if 7.2 c.c. are drawn off, 1.2 c.c. of saline are put in the bottle. The contents are now well mixed and transferred by means of long pipettes to sterile Wintrobe tubes (3 or 4 are required), which are closed with rubber caps when full. These tubes are centrifuged at 2000 r.p.m. for 20 minutes. The supernatant fluid is pipetted off and the white "buffy coat" transferred into fresh Wintrobe tubes, which are filled up with saline, capped and centrifuged at 2000 r.p.m. for another 15 minutes. The supernatant layer is withdrawn and the white layers pipetted off into a 10 c.c. bottle containing 4 c.c. of saline. A count of the nucleated cells in this suspension is made. There are usually more than 10,000 per c.mm., so saline is added to give this final concentration.

Into each culture bottle is now put 0.5 c.c. of marrow cell suspension, 0.75 c.c. saline and 1.25 c.c. placental serum. This gives a final concentration of 2000 cells per c.mm. in 50 per cent. placental serum; the proportions of the fluid to gas space are within those suggested by Parker. From a suitable gas reservoir a mixture of 50 per cent. oxygen and 3.5 per cent. CO₂ is passed for 2 minutes through 1 per cent. CuSO₄ solution, then through a tube about 3 cm. long packed with sterile wool, and finally through a bent pipette into the culture bottles. The bottle is capped, sealed with celloidin solution, marked and put in the incubator at 38° C. Usually there is enough marrow suspension to make 8-12 cultures.

Withdrawal of specimens for examination. The bottle is well shaken, the rubber cap taken off and the neck flamed. With a pipette about 1 c.c. is withdrawn and put in a watch glass which is covered. The standard gas mixture is passed through the bottle for 1 minute, the neck flamed, a fresh rubber cap put on and sealed and the bottle replaced in the incubator.

From the fluid in the watch glass a nuclear cell count can be made and the rest is transferred to a Wintrobe tube and centrifuged at 2000 *r.p.m.* for 15 minutes. Practically the whole of the supernatant fluid is pipetted off, only leaving an amount about equal to the small white layer at the bottom of the tube; this layer is thoroughly mixed with the remaining fluid and withdrawn. Large drops are placed on slides and smears are made from them. These are allowed to dry and then stained as follows. Stain with Jenner's stain, 2 minutes; dilute with an equal part of dilution fluid, 1 minute; pour off and without washing add Giemsa solution and stain for 3 minutes. It is useful to differentiate half the slides with fresh 1:1500 acetic acid. The dilution fluid is a phosphate-NaOH buffer of pH 6.4 and the Giemsa solution is made by adding 1 drop of Giemsa stain to each c.c. of dilution fluid. The slides are examined first with a 4.3 mm. oil-immersion objective and then with a 2 mm. lens for further detail.

Changing the medium. This is done every second day but can be left 3 days if necessary after the first change. The bottles are centrifuged at 3000 *r.p.m.* for 15 minutes. If satisfactory separation has not occurred the speed is increased to 4500 *r.p.m.* for the final 2 minutes. As much of the supernatant fluid as can be withdrawn without disturbing the cellular layer is transferred to a measuring cylinder. The volume is replaced half with saline and half with placental serum. Gas mixture is passed for 1 minute through the bottle, which is then recapped, sealed and replaced in the incubator.

Results

Marrow for culture containing large numbers of leucocytes of one particular group was obtained from patients with various types of leukæmia. In this way practically pure cultures of granulocytes, lymphocytes and monocytes were obtained. Leukæmia is a pathological condition, but the marrow leucocytes in this condition seem to follow what have been regarded as normal modes of development.

Granulocytes (figs. 1-6). The marrow was obtained from patients with chronic myeloid leukæmia. Most of the cells were polymorphonuclears, metamyelocytes and myelocytes, with some myeloblasts. In the case illustrated the percentages of the various cells present were:—

Polymorphonuclears	. 53.2	Myeloblasts.	. 1.2
Eosinophils 4.2	Normoblasts "B"	1.4
Basophils 1.6	., "C"	2.2
Metamyelocytes . .	. 14.0	Lymphocytes .	2.2
Myelocytes 20.0		

Mitotic figures were seen in the myelocytes.

After 24 hours' culture the marrow cells appeared little changed except that some of the polymorphs were degenerate. Mitotic figures in all stages from prophase to telophase were seen in the myelocytes, both in the early forms with undifferentiated granules (promyelocytes) and in those with well differentiated neutrophil

GRANULOCYTE CULTURES (figs. 1-6). LYMPHOCYTE CULTURES (figs. 7 and 8)

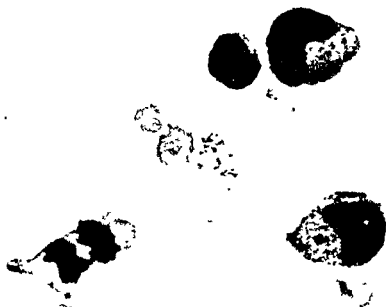


FIG. 1.—2nd day. Neutrophil myelocyte in telophase and an eosinophil myelocyte.



FIG. 2.—3rd day. Developing neutrophil polymorphonuclears



FIG. 3.—4th day. Neutrophil myelocyte in metaphase

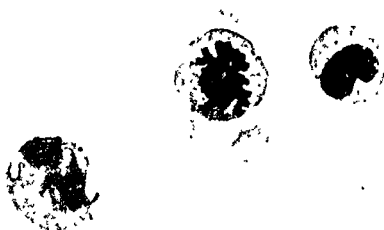


FIG. 4.—4th day. Neutrophil myelocyte in early metaphase and metamyelocytes



FIG. 5.—7th day. Neutrophil myelocyte in early metaphase



FIG. 6.—7th day. Myelocytes showing irregular shapes.

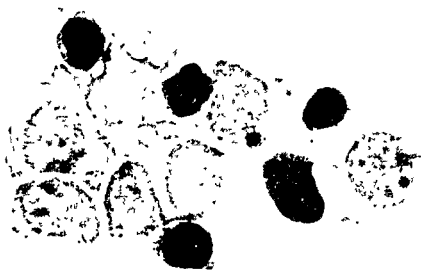


FIG. 7.—2nd day. Lymphoblasts and large and small lymphocytes.



FIG. 8.—7th day. Lymphoblast and small lymphocyte

granules. The outlines of the cells were rather irregular and vacuolation of their cytoplasm was seen. The myeloblasts could be distinguished by their short grape-like protrusions of cytoplasm. The second day showed a very similar picture ; signs of commencing nuclear lobulation were seen in some of the metamyelocytes. In the 3rd day cultures the intermediate stages between myelocytes, metamyelocytes and young polymorphs were numerous. The nucleus becomes incised or kidney-shaped, then the nuclear material becomes concentrated at what are presumably the sites of the future lobes. Many of these developing cells have very oddly shaped nuclei. The 4th day cultures showed similar changes, but the polymorphs were all degenerate and pyknotic. Myeloblasts were very few. Throughout these days myelocytes in mitosis were present in various stages. By the 7th day the degenerate polymorphs were much fewer and some healthy ones had appeared ; the metamyelocytes nearly all had curiously shaped nuclei ; the myelocyte nuclei were also irregular but not lobed. Mitoses were still present in the myelocytes. The 10th day cultures showed a similar picture but by the 14th day degeneration had set in, the cells were fewer, nuclei smaller and cytoplasm vacuolated. No mitoses were found.

Lymphocytes (figs. 7 and 8).—The marrow was taken from patients with chronic and subacute lymphatic leukaemia. The cells were mostly lymphocytes with some lymphoblasts ; in the case illustrated the percentages were :—

Polymorphonuclears	2.0
Lymphocytes	78.0
Lymphoblasts	19.0
Normoblasts " C "	1.0

In the 2nd day cultures there were typical small lymphocytes, large lymphocytes with fairly abundant grey-blue cytoplasm and large lymphoblasts with well-marked nucleoli. Many of the large lymphocytes showed the single pseudopodium characteristic of motile cells. No mitotic figures were found, but double or partially separated nuclei denoting amitotic division were fairly frequent. This picture remained more or less unchanged ; lymphoblasts were but few by the 7th day and many of the small lymphocytes were degenerate. Increasing degeneration occurred in the older cultures.

Monocytes (figs. 9-18). Two patients with monocytic leukaemia were available for study and both sets of cultures gave identical results. In the case illustrated 32.4 per cent. of the marrow cells were mature monocytes, 60 per cent. immature monocytes and monoblasts, the remainder being occasional granulocytes, normoblasts, lymphocytes and plasma cells.

In the 2nd day cultures the monocytes showed an astonishing variety of size and shape, irregularity of outline of both nucleus

and cytoplasm being notable. The monoblasts remained round but there were usually two or three grape-like protrusions of cytoplasm. Many of the monocytes had red granules in the cytoplasm and when mitosis occurred the cytoplasm became foamy and the red granules more prominent. In the 4th day cultures the monocytes were actively phagocytic, had engulfed debris from degenerate cells and showed large fat vacuoles. Some of the nuclei were slightly lobulated. Mitotic figures at various stages were seen in the monoblasts and early monocytes. The 7th day cultures were similar but cells were fewer and there were more degenerate cells: monoblasts were very few. By the 9th day there were two chief varieties of cells: (A) small cells, elongated, with dark granulated cytoplasm and only small, if any, vacuoles; (B) larger cells with abundant cytoplasm containing much phagocytosed debris and fat. Type B seems to be produced from type A by phagocytosis, since intermediate forms occur. These cells remained the characteristic feature of the cultures and were seen up to the 21st day, when only a few were left.

Discussion

The results suggest that under the conditions of these experiments granulocytes, lymphocytes and monocytes follow quite distinct lines of development. The granulocytes show mitosis up to and including the stage of myelocyte with differentiated granules, further development proceeding by lobulation of the nucleus. Osgood (1937) found that polymorphonuclears from blood survive in culture on the average only 61 hours, and that, as in these experiments, fresh polymorphonuclears appear in the marrow cultures long after this period, presumably having developed from the metamyelocytes. Lymphocytes remain more or less unchanged; they do not show mitosis but amitotic division occurs. The monocytes develop into actively phagocytic cells and the early forms, including those with granules in the cytoplasm, show active mitosis.

So far as could be seen forms intermediate between the three types of leucocyte did not occur. Phagocytic macrophages did not appear in the granulocyte or lymphocyte cultures.

In all types of cultures the early "blast" cells become steadily fewer and it is probable that they mature into later forms, since this diminution occurs at a period when only fully mature cells are showing degeneration.

Summary

1. A method for the culture *in vitro* of human bone marrow cells is described in detail.

2. Cultures of bone marrow showing hyperplasia of the three chief groups of leucocytes—granulocytes, lymphocytes and monocytes—have been studied.

MONOCYTE CULTURES



FIG 9—1st day Mono-
cyte in telophase,
note granular foamy
cytoplasm

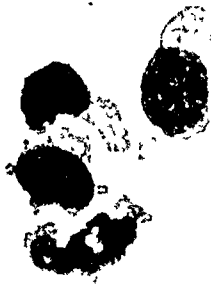


FIG 10—2nd day Monocytes
showing very irregular shapes
and a monoblast



FIG 11—2nd day Ir-
regularly shaped mono-
cytes and an early
monocyte in prophase

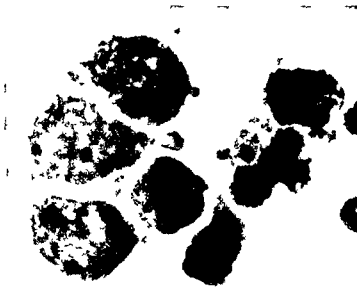


FIG 12—2nd day Monoblasts, irregularly
shaped monocytes and a myelocyte



FIG 13—4th day Phagocytic monocytes



FIG 14—7th day Monocyte
in early anaphase



FIG 15

FIG 15—7th day



FIG 16

FIG 16—9th day



FIG 17

FIG 17—9th day Type B monocyte



FIG 18

FIG 18—21st day Phagocytic monocyte

3. These three groups show characteristic differences under the conditions of the experiments and intermediate forms do not occur : the three groups appear to have distinct modes of development.

I am indebted to Dr John F. Wilkinson and the other members of the Honorary Staff of the Manchester Royal Infirmary who have given me access to their patients, and to the Medical Superintendent of Withington (Manchester City) Hospital for facilities in the labour wards.

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SHORT ARTICLES

616—002 5 616—036 1

INFLUENCE OF THE SIZE OF THE INFECTING DOSE ON THE COURSE OF TUBERCULOSIS

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(PLATE XXI)

Physicians are prone to stress the importance of differences in the infecting dose in the initiation and course of tuberculosis though clinical observation alone does not provide a basis for a sufficiently exact evaluation of the size of the infecting dose.

In our work on infants and children of pre school age at the tuberculosis centre we tried to estimate the size and frequency of the infecting dose on the basis of the numbers of bacilli found at different times in the sputum of the sick person who was the source of infection and the frequency and intimacy of the tuberculous patient's contact with the child at the time. It turned out that the intensity of infection defined in this way could not be correlated with the subsequent history of the child. This method of investigation proved quite inadequate for evaluating the influence of the dose on the child's health.

Even animal experiment has led to contradictory conclusions on this subject. On the basis of experiments on rabbits and guinea pigs, Calmette, Boquet and Negre (1923) came to the conclusion that the survival time of the experimental rabbit is inversely proportional to the quantity of bacilli injected. These authors do not give the number of animals used in their experiments and speak only of 'numerous animals', so that the operation of chance cannot be excluded. The differences moreover are small and statistically not significant. Lange (1931, 1934) on the other hand, believes, on the basis of the number of primary foci produced in the lungs of guinea pigs infected by inhalation that in tuberculosis there almost always occurs an *infectio minima*, small differences in the number of bacilli cannot have a decisive influence on the course of tuberculosis, which is determined by natural resistance.

During our investigations of experimental tuberculosis in the guinea pig we came to the conclusion that the best criterion of severity is the mean survival time in sufficiently large groups of animals. So far as possible we have used in each group 10 guinea pigs of the same age and weight from a highly inbred strain and have tried to throw some light on the problem of the influence of the size of the infecting dose on the course of guinea pig tuberculosis.

Experiment 1 (row 1 in table). Ten guinea pigs were inoculated subcutaneously in the left hind leg with 10^2 mg of bovine bacilli (1714) and 10 others with 10^1 mg. The survival times did not differ significantly.

TABLE
Effect of dosage on survival time in tuberculosis of guinea-pigs

Experiment	Guinea-pigs inoculated	Strain	Dose (mg.)	Guinea-pigs included in calculation	Limits of survival of guinea-pigs which died from tuberculosis (days)	Mean survival time (days), with standard error	Standard deviation (days)	P	Is the difference significant?
1 a	10	1714	10^{-2}	10	81-144	102 ± 5.6	17.8	>0.6	No
b	10	1714	10^{-1}	10	69-136	$98 \pm$	24.9
2 a	10	1714	10^{-2}	10	47-163	96 ± 11.3	35.7	<0.05	a and b: Yes
b	10	1714	7×10^{-4}	10	54-198	137 ± 14.4	45.6	>0.1	b and c: No
c	10	1714	10^{-4}	9	69-233	122 ± 17.2	52.1	>0.2	a and c: No
3 a	17	1714	10^{-2}	17	90-206	147 ± 8.5	35.3	<0.01	a and b: Yes
b	17	1714	7×10^{-6}	14	102-378	234 ± 21.8	81.3	>0.3	b and c: No
c	17	1714	10^{-6}	8	200-379	262 ± 18.8	53.3	<0.01	a and c: Yes
4 a	10	1714	10^{-2}	10	123-264	174 ± 12.3	39.1
b	10	1714	23×10^{-6}	2
c	10	1714	10^{-6}	0
5 a	9	705	10^{-1}	9	56-163	88 ± 11.7	35.1
b	9	705	10^{-1}	9	78-304	159 ± 25.3	76.1	<0.05	Yes

INFECTING DOSE AND COURSE OF TUBERCULOSIS



FIG. 1—Tuberculosis of tracheo bronchial lymph nodes of guinea pig infected with 10^{-6} mg of tubercle bacilli and killed after 423 days. No tuberculous granulation; the dark fibres are fuchsinophil.



FIG. 2—Fibrous tuberculosis of lumbar lymph node of guinea pig infected with 10^{-6} mg of bacilli and killed after 367 days, showing fibrous and epithelioid tubercles with a fibrous margin.



FIG. 3.—High-power view of part of fig. 2

Experiment 2 (row 2 in table) Ten guinea pigs (a) received 10^{-2} mg of bovine tubercle bacilli (1714) in the left hind leg, 10 others (c) a dose 100 times smaller (10^{-4} mg), and a third group of 10 (b) the same dose (10^{-4} mg) 7 times at 2 day intervals subcutaneously in various parts of the abdomen and legs.

For groups (a) and (c) the difference of means is not significant according to Fisher's formula (1936). The difference between groups (c) and (b) is likewise not significant but that between the means of groups (a) and (b) is significant ($P < 0.05$). The influence of a hundredfold difference in the infecting dose is marked, though inconstant and of doubtful significance.

Experiment 3 (row 3 in table) Seventeen guinea pigs (a) were injected subcutaneously in the left hind leg with 10^{-2} mg of bacilli from culture 1714, 17 (c) with a dose 10 000 times smaller (10^{-6} mg), and a third group of 17 (b) received the same dose (10^{-6} mg) 7 times daily subcutaneously in the abdomen and legs. In group (b) one guinea pig died prematurely from purulent pericarditis and pneumonia. Of 2 killed 400 days after infection, one showed limited healing tuberculosis of lymph nodes and the second generalised disseminated tuberculosis. In group (c) only 8 died from tuberculosis, four died from other causes after more than 350 days and showed only limited and healing tuberculosis of the lymph nodes, as did three of the 5 animals killed 400 days after infection. The 2 others showed no lesions at all.

The tuberculosis, limited to the lymph nodes, was characterised by a prominent growth of fibrous tissue which was partly hyaline and sometimes without traces of tuberculous granulation tissue. Figs 1-3 illustrate very well the fibrous character of this rare form of tuberculosis in guinea pigs. The survival times of the various groups, excluding the animals which died accidentally or were killed after 400 days, are given in row 3 of the table. The differences between (a) and (b) and between (a) and (c) are significant, that between (b) and (c) is not significant.

Experiment 4 (row 4 in table) Ten guinea pigs received 10^{-2} mg of bacilli of culture 1714, 10 others 10^{-6} mg, and a third group of 10 received 23 daily doses of 10^{-6} mg of a recently prepared emulsion of a 3 weeks culture.

The results of this experiment cannot be treated statistically. The animals in the group which received the dose of 10^{-2} mg died from tuberculosis in 123-264 days (mean 174 ± 12.3). In the group which received 23 doses of 10^{-6} mg two died from tuberculosis (191 and 216 days) and one from pneumonia. The remaining animals were killed at 383 days, 4 showed limited healing glandular tuberculosis and 3 no tuberculous lesions at all. In the group which received only one dose of 10^{-6} mg two died but not from tuberculosis, of 8 killed at 383 days only one showed limited healing tuberculosis of lymph nodes, the remainder had no tuberculous lesions at all.

The difference in dosage here had a distinct effect. The larger dose (10^{-2} mg) was regularly fatal, whereas the smaller dose of 10^{-6} mg did not cause progressive tuberculosis and even when given 23 times caused generalised tuberculosis in only 2 out of 10 guinea pigs.

Experiment 5 (row 5 in table) In this experiment we used a human strain—705, isolated in 1937 from a fatal case of tuberculosis in a child. Nine guinea pigs received 10^{-1} mg of culture in the left hind leg subcutaneously, and 9 others 10^{-4} mg. All died from tuberculosis after varying intervals of time.

The influence of the thousandfold difference in infecting dose is quite clear in this experiment. The difference of the mean survival times is statistically significant.

The effect of differences in the size of the infecting dose on the subsequent course of tuberculosis in the guinea-pig may be summarised as follows.

Difference in infecting dose	Difference in course of tuberculosis	Demonstrated in experiment
10-fold	None	1
100-fold	Marked but uncertain	2
1000-fold	Significant	5
10,000-fold	„	3

SUMMARY

Differences in the size of the infecting dose exert an influence on the course of experimental tuberculosis in the guinea-pig only when they are very great.

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INFLUENCE OF THE VIRULENCE OF INFECTING BACILLI ON THE COURSE OF TUBERCULOSIS

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The influence of the virulence of the infecting organism on the course of tuberculosis is not yet definitely settled. It is known that in some cases of skin tuberculosis the virulence of the bacilli can be low (Griffith, 1930). In our work on this problem we were mainly interested in the question as to whether the varying course of lung tuberculosis in man depends in some degree on differences in virulence of tubercle bacilli.

In their attempts to investigate the relation between the virulence of tubercle bacilli for guinea-pigs and the clinical form of pulmonary tuberculosis in man, Bergeron and his collaborators (Bergeron and Mézière, 1936; Bergeron and Normand, 1938) injected 0.2 mg. of each strain into one guinea-pig and estimated virulence on the basis of survival time. They came to the conclusion that virulence runs parallel to the clinical state of the patients. It seems clear that the method of estimating virulence is unsuitable because it does not exclude the role of chance. Lange (1930)

and those who have used his method (Opitz and Sherif, 1929, Roloff and Pagel, 1929, de Carvalho, 1932-33) have made better virulence determinations than were made previously. Lange injected graded minimal doses of various strains intracutaneously into groups of guinea pigs and endeavoured to determine the number of bacilli injected by sowing the same quantity of the emulsion on media. He determined the time of appearance of the primary lesion in the infected animals and the extent of the tuberculous lesions at autopsy after 3 months. For economy Roloff and Pagel, and de Carvalho used a modification of this method (Lange and Lydén, 1928), injecting equal doses of four strains into the same animal. Lange came to the conclusion that the virulence determined in experiments on animals does not correspond at all to the state of the patient from whom the strain was cultivated, Opitz and Sherif that the clinical form of tuberculosis in children does not depend on virulence, Roloff and Pagel that there is no connection between the virulence of bacilli cultivated from the sputum of patients with open pulmonary tuberculosis and the form and course of the disease and de Carvalho that the bacilli in the sputum of different patients have approximately equal virulence. The authors who use the "four corner method" realise that the saving in animals is gained at the sacrifice of exactness and Lange and de Carvalho point out that one can estimate virulence only by observing the course of the disease in infected animals.

In all our experiments with one exception we used guinea pigs of the same age from the same highly inbred strain, and estimated the degree of tuberculosis on the basis of survival time combined with post mortem examination to ascertain the cause of death. We do not share Lange's apprehension as to the frequent dependence of the time of death not on the evolution of the tuberculosis but on casual infections, in a well organised animal house these possibilities do not play an important role in influencing the course of experiments. The post mortem macroscopic (and in doubtful cases microscopic) examination protects us from errors.

We were obliged to restrict our experiments to the simultaneous comparison of only two strains, because we were unable to obtain more than 20-30 animals of the same age at once. In order to ascertain how far virulence for guinea pigs corresponds to the clinical course in human beings, we examined two strains in each experiment, one from a mild and the other from a fatal case of tuberculosis. In four experiments doses of 10^{-4} mg and in one a dose of 10^{-2} mg of culture were inoculated into each of a group of guinea pigs.

Experiment 1 (table) Fifteen guinea pigs were injected subcutaneously in the left hind leg with 10^{-4} mg of a 3 weeks old culture of the bovine strain 311, obtained after 3 passages through guinea pigs from a case of tuberculosis verrucosa cutis in an adult and cultivated for 21 months on egg media. A second group of 15 received in the same way the same dose of a 3 weeks old culture of a bovine strain 542, obtained after one guinea pig passage from the gastric lavage of a 7 year old boy who died from chronic disseminated milary tuberculosis, the post mortem examination did not show a distinct primary lesion though there were some tuberculous ulcers in the ileum. This strain had been cultivated on artificial media for 8 months. One animal in the first group died from pneumonia and one from a purulent polyserositis. All the others died from disseminated tuberculosis.

One may conclude from the findings that the strain isolated from the skin condition is less virulent than that from the fatal case, these findings conform with Griffith's results.

Experiment 2 (table) Sixteen guinea pigs received subcutaneously in

TABLE

Survival times of guinea-pigs inoculated with different strains of tubercle bacilli

Experiment	Guinea-pigs inoculated	Strain	Dose (mg.)	Guinea-pigs included in calculation	Limits of survival of guinea-pigs which died from tuberculosis (days)	Mean survival time (days), with standard error	Standard deviation (days)	P	Is the difference significant?
1	15	311 (bovine). Tuberculosis verrucosa cutis	10 ⁻⁴	13	79-181	120 ± 7.7	26.5	<0.01	Yes
	15	542 (bovine). Disseminated milary tuberculosis	10 ⁻⁴	15	70-129	96 ± 4.3	16.4		
2	16	155 (human). Primary lung complex, healed afterwards	10 ⁻⁴	15	157-600	291 ± 27.8	107.3	<0.01	Yes
	16	473 (human). Primary lung complex and fatal milary tuberculosis	10 ⁻⁴	15	113-258	183 ± 11.1	43.0		
3	10	640 (human). Chronic pulmonary tuberculosis	10 ⁻⁴	9	68-214	121 ± 14.7	44.1	>0.1	No
	10	670 (human). Fatal generalised milary tuberculosis	10 ⁻⁴	9	94-259	138 ± 17.2	51.6		
4	10	673 (human). Lung infiltration	10 ⁻⁴	10	47-139	93 ± 9.7	30.5	<0.05	Yes
	10	647 (human). Fatal milary tuberculosis	10 ⁻⁴	10	61-372	157 ± 28.2	89.1		
5	10	752. Lung infiltration	10 ⁻²	10	78-206	117 ± 11.4	36.0	>0.1	No
	10	746. Fatal milary tuberculosis	10 ⁻²	10	49-148	103 ± 8.2	25.8		

the left hind leg 10^{-4} mg of a 3 weeks old culture of a human strain 155, cultivated directly from the gastric lavage of an infant with a primary lung complex which afterwards healed completely. This strain had been cultivated for 33 months on artificial media. Sixteen others received in the same way the same dose of a 3 weeks old culture of a human strain 473, cultivated directly from a tracheo bronchial gland taken during autopsy in a 4 year old girl who died from disseminated miliary tuberculosis following the primary pulmonary complex. This strain was cultivated on media for 14 months. In each of these two groups one non specific death occurred, while the remaining animals died from tuberculosis.

The results (expt 2 in the table) seem to suggest a conformity between the course of the disease and the virulence of the strains, but these strains differ notably as to the period of cultivation on artificial media. Strain 155, which was less virulent in these experiments, had been cultivated for 24 years, strain 473 for one year only. From our own observation we have learnt contrary to the opinion of some authors (Opitz and Sherif), that long cultivation on artificial media without animal passage is apt to diminish virulence. In view of the result of expt 2, and because of the impossibility of comparing the primary strains, we used in subsequent experiments only recently isolated strains, cultivated for the same length of time on artificial media.

Experiment 3 Ten guinea pigs received subcutaneously in the left hind leg 10^{-4} mg of the human strain 640, cultivated directly from the sputum of an 11 year old girl who had pulmonary tuberculosis of the adult type and who was treated by a bilateral pneumothorax. After more than 2 years she now shows a progression of her lesions associated with puberty. This strain had been growing on artificial media for 3 months. Ten other guinea pigs received in the same way the same dose of a culture of human strain 670, obtained after one passage through a guinea pig from the gastric lavage of a 12 year old girl who died from disseminated miliary tuberculosis. This strain had been growing on artificial media for 1 month.

In each group one non specific death occurred. The table (expt 3) shows the survival times of the remaining animals, all of which died from tuberculosis. These two human strains, derived from two entirely different forms of tuberculosis in girls of the same age, did not show significant differences in virulence in experiments on guinea pigs.

Experiment 4 Ten guinea pigs received subcutaneously in the left hind leg 10^{-4} mg of a 2 weeks old culture of the human strain 673, obtained after one guinea pig passage from the gastric lavage of a 10 year old girl with infiltration of the hilus of the right lung which healed after more than 2 years' observation, leaving only small areas of calcification. This strain had been growing on artificial media for 3 months. Ten other guinea pigs received in the same way the same dose of a 2 weeks old culture of the human strain 647, isolated directly from a tracheo bronchial gland taken during the post mortem investigation of a 5 year old girl, who died from miliary tuberculosis following a pulmonary primary complex. This strain had been cultivated on media for 4 months.

All these guinea pigs died from tuberculosis. Their survival times are shown in the table (expt 4). In this experiment the strain isolated from fatal miliary tuberculosis showed lower virulence for guinea pigs than that isolated from a case of lung infiltration which afterwards healed.

Experiment 5 Ten guinea pigs received subcutaneously in the left hind leg 10^{-2} mg of the human strain 752, isolated after one guinea pig passage from the gastric lavage of a 2 year old boy with a small lung infiltration of the right hilus which healed afterwards. This strain had been growing on artificial media for 1 month. Ten others received in the same

way the same dose of a 2-weeks-old culture of the human strain 746, obtained from a tracheo-bronchial gland taken during the post-mortem investigation of a 3-year-old girl who had died from generalised miliary tuberculosis following primary lung tuberculosis. This strain had been growing on media for 2½ months.

All these guinea-pigs died from tuberculosis and their survival times are shown in the table (expt. 5). In this experiment both strains showed essentially the same virulence for guinea-pigs.

Summary

In these experiments there appears to be no correlation between the course of the disease in the human subjects from whom the strains of tubercle bacilli were obtained and the virulence for guinea-pigs of the organisms isolated, except in the case of lupus.

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THE CHARACTERISTIC HAPTENE AND ANTIGEN OF RUGOSE RACES OF CHOLERA AND EL TOR VIBRIOS

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In an earlier note on rugose variants of vibrios (White, 1938) the question of the serological activity of the gelatinous intercellular substance characteristic of such cultures was left open. It has now been shown that this material consists of, or contains, a hapten which reacts specifically with the antiserum of the whole rugose culture and with the antisera of certain extracts of rugose vibrios.

The rugose cultures mainly studied have been derived from S, R and ρ cultures of the cholera strains "Rangoon," "Inaba" (both well known from the recent literature) and "Shanghai 10" (obtained in 1934 through Dr Y. N. Yang) and from S and R cultures of the El Tor strains "1934-D11" and "1934-W1," received from Dr Doorembos. The rugose races were not isolated by any particular method but were selected wherever opportunity

offered from ordinary laboratory cultures, from platings of cultures at the limit of viability and from platings of mixtures prepared in specific bactericidal tests. The variants were maintained by reselection. My own rugose derivatives of "Rangoon S" were supplemented by a single cell rugose race of the same strain received from Dr R. W. Linton under the label "Rangoon 3a R 2". Both El Tor strains, like so many El Tor cultures, had, both in the S and R form, a predilection for the rugose growth habit and it was difficult to obtain strictly non rugose races from them for comparative work.

The 18-24 hour agar cultures were carefully scraped from the medium and emulsified as far as possible in saline, about 800 c.c. to the growth from 10 sq. ft. of agar surface, a little chloroform was added as preservative. The mixture having stood overnight at room temperature, the bacterial mass was separated by centrifugation from the supernatant fluid, which normally contained little or none of the haptene in question and was turned to other purposes. With or without a further period of extraction with saline at room temperature the bacterial mass was re-suspended in saline and briefly heated in a boiling water bath. After cooling, the mixture was acidulated with acetic acid and centrifuged to separate the culture mass from the supernatant fluid, which contained, with a part of the rugose specific substance, most of the agar contaminating the material. The culture mass was again returned to saline and, the reaction having been made neutral, the suspension heated for $\frac{1}{2}$ hour on the water bath. Then as before the mixture was cooled, acidified with acetic acid and centrifuged. This process was repeated till the yield of specific substance was inappreciable. The acid extracts were filtered clear and then usually, though not of necessity, neutralised. With shaking to ensure the presence of air bubbles, the extracts were suddenly mixed with two volumes of alcohol. When present in high concentration, as in the earlier extracts, the rugose specific substance was at once precipitated as a massive but rapidly contracting gelatinous clot, which, charged with air bubbles, rose to the surface and could be pressed to a dough-like mass and transferred in its entirety to another vessel. From the later, less concentrated extracts the reactive substance was precipitated as diaphanous gelatinous floccules which could be gathered with a glass rod into a single mass. Finer more slowly forming precipitates were discarded. The precipitates, often pooled, were re-dissolved in sufficient boiling water or saline. The process of solution was often protracted, particularly where the precipitates had been subjected to pressing and drying. The solution was acidified (N/50) with acetic acid and heated on the water bath till no further precipitation occurred. The precipitate was removed and the rugose haptene was re-precipitated with alcohol as before from the supernatant fluid.

Variations on this process were (a) to heat the primary hot saline extracts with N/50 N/20 acetic acid and filter before proceeding to precipitation with alcohol, (b) to extract the bacterial mass with N/50 N/20 acetic acid, and (c) to precipitate the specific substance with ammonium sulphate at half saturation, either in place of or as a sequel to precipitation with alcohol.

The treatment of the extracts at one stage or another with hot dilute acetic acid served to precipitate rough antigens present and, during precipitation and re-precipitation of the rugose haptene, smooth specific substances were gradually lost. Fractions obtained during the earlier stages of extraction gave precipitates with an agar precipitating serum kindly furnished by Dr W. T. J. Morgan, the later fractions gave none and were presumably agar free.

The preparations gave a negative biuret test but an intense Molisch reaction, best observed by placing the layered reaction tubes for a few moments in a bath of boiling water.

Potent precipitating antisera for the rugose specific substance were prepared by immunisation of rabbits with S, R and ρ rugose cultures of cholera and El Tor vibrios—living, killed at 56° C. or heated at 100° C. None of these sera gave precipitation with agar.

Rugose variants of S cultures yielded a fraction contaminated, at least in the first place, with the smooth specific haptene and stimulated in rabbits both anti-rugose and anti-smooth precipitins and agglutinins. Rugose R and ρ races on the other hand readily supplied the rugose specific substance in a state of serological purity and their antisera could be used for the detection of the rugose substance wherever present.

Rugose antisera were also successfully prepared against boiled and filtered neutral solutions of the acetic acid precipitates from saline extracts of rugose cultures prepared at room temperature and autolysed during several days at 37° C.

The rugose antigen, it seems, is heat-stable and may occur in solution as an acid-precipitable complex. Since the preparations obtained have been contaminated with other antigens no more precise statement as to their character is possible.

None of the very many cholera antisera prepared against ordinary non-rugose cultures showed appreciable precipitins for the rugose specific substance, though some possibly contained traces.

Eight rabbits which each received over a month 7 or 8 intravenous injections of different samples of the biuret-negative rugose specific substance failed to develop precipitins for that substance. Several others inoculated with crude preparations extracted with hot saline from young cultures but not heated in acid solution also gave an inappreciable response.

In ring precipitation tests with undiluted anti-rugose serum the rugose specific substance reacted in a characteristic manner, forming at the interface a coherent disc which, soon contracting, fell as a clot to the bottom of the tube; about the interface a secondary floccular precipitate formed more slowly. Visible reactions were obtained with solutions of the specific substance as dilute as 1 : 500,000 and 1 : 1,000,000.

Where the reagents were mixed in equal parts the precipitates formed were diaphanous and floccular, not unlike those of agglutinated flagella. In optimal mixtures the floccules condensed to a coherent pad at the bottom of the tube.

The precipitation reaction was not appreciably altered in character or titre when the specific substance was treated for 15 minutes at 100° C. with *N*/10 acetic acid or *N*/10 NaOH.

The taxonomic specificity of the rugose substance has not yet been extensively investigated. It can be said that the samples isolated from the Inaba and Ogawa types of cholera and El Tor vibrios cannot be differentiated by precipitation tests and that such fixation-of-precipitin tests as have been made suggest that they are serologically identical. It is clear, too, that the rugose substance is not affected by S-R- ρ variation.

Samples of rugose substance extracted from vibrios belonging to groups other than O group I of Gardner and Venkatraman (1935) have reacted variously with anti-rugose cholera-El Tor sera. To take only the case of certain strains with a place in recent literature (White, 1934; White and Yang, 1934; Linton and Mitra, 1934-35; Linton, Shrivastava and Mitra, 1934-35 *a* and *b*; Gardner and Venkatraman, 1935; Linton, 1935; Linton, Mitra and Mullick, 1935-36; Linton, Mitra and Seal, 1935-36; Taylor and Ahuja, 1935-36 *a* and *b*; Seal, 1936-37; Linton, Seal and Mitra, 1937-38), preparations from rugose races of the water strains—Nanking 1932-109 and 110 (both O group IV) and Nanking 1932-101 ("individual O")—have

reacted as strongly as the homologous group I preparations, those from the faecal strain Kasauli 73 (O group VI) and the water vibrio W 880 have given positive but less pronounced reactions, while those from Nanking 1932 77 and 126 (both O group II from cholera stools), Nanking 1932 123 (O group III from cholera), the water strain Kohat and the cultures "Rangoon rough 1," and "Rangoon rough 2" of Linton, have given negative reactions.

The negative results obtained with extracts of a rugose derivative of "Rangoon rough 1," which is a smooth culture with the serology of *V. melchnikovi*, and of the capsulated culture "Rangoon rough 2" support my contention (White, 1937, 1940) that these strains have no immediate relation to nor derivation from their alleged parent, the cholera strain "Rangoon smooth." It was shown by cross precipitation tests in both directions that no significant serological similarity exists between the materials which confer the rugose growth character on "Rangoon rough 2" and on the single cell culture "Rangoon 3a R 2" later derived by Linton, Seal and Mitra from "Rangoon smooth." "Rangoon 3a R 2" remains, in spite of its superimposed rugose character, a typical S race of *V. cholerae*.

Usable if not ideal suspensions of rugose cultures for agglutination tests were prepared by grinding the growths with saline. By using living smooth rugose suspensions and antisera prepared against rough rugose cultures, heated at 100° C to inactivate flagellar antigen, optimal conditions for the study of the uncomplicated rugose agglutination reaction were obtained. Similar "isolation" of this reaction was achieved by the use of S and R anti rugose sera which had been saturated with non rugose cultures.

Suitable sera which failed to clump the appropriate S or R non rugose suspension at 1:20 and higher dilutions caused slow partial clumping of the corresponding rugose suspensions in loosely floccular masses when incubated at 45-50° C for 3-4 hours and at room temperature overnight. The titre of specific clumping was not always easy to ascertain owing to gradual sedimentation of the suspended particles. Attempts to improve the test suspensions by centrifugation removed the reactive material. The agglutination observed is almost certainly a clumping of particles of intercellular substance containing vibrios rather than of dispersed vibrios.

The readings obtained with a rugose antiserum and suspensions made at different times from a single rugose strain, or even at different stages in the growth of a single rugose culture, were erratic, varying in some cases between 1:100 and 1:1000. These irregularities are possibly related to autolytic changes.

In agglutination tests with antirugose cholera sera and rugose races of heterologous vibrios results followed precisely the indications of the corresponding precipitation tests: only cultures yielding precipitable extracts were agglutinated.

Though leaving something to be desired in technique, the observations show that there is a definite rugose agglutination reaction and disclose a flaw, actual if in practice unimportant, in the doctrine of the serological specificity of the heat stable agglutinogens of O group I vibrios.

It is difficult to escape the conclusion that the rugose substance is a protective secretion with a role in assisting the survival of the race in nature. In the laboratory it affords defence against unfavourable conditions and the action of serum: it has repeatedly been observed that rugose forms tend to grow out, often in pure culture, from mixtures made in specific bactericidal tests and to survive their associates in ageing cultures of vibrios. In view of the possibility that rugose vibrios might resist the acid conditions of the stomach and have a special role in infection, their relative resistance to hydrochloric acid in saline and broth media has been tested: on the whole

rugose races of *V. cholerae* have failed to show any strikingly enhanced resistance to acid. Rugose races appear to be not infrequently isolated from cholera cases. I have received several such cultures, obtained from cholera stools, as alleged rough variants. It is possible that the addition of rugose culture to the mixture of strains used in preparing cholera vaccines might be advantageous.

Summary

From S, R and ρ rugose cultures of cholera and El Tor vibrios there may be isolated in relatively large amount, by methods described, a common non-protein carbohydrate-containing haptene absent from or inconspicuous in non-rugose cultures. This substance reacts by precipitation in high dilution and in a characteristic manner with antisera of whole rugose cultures and of certain extracts obtainable from them. Corresponding with this precipitation there is a rugose agglutination reaction. The rugose antibodies of O group I vibrios agglutinate rugose races of certain vibrios belonging to other O groups defined by Gardner and Venkatraman and give intense precipitation with their rugose haptenes. The rugose antigen resists heating in neutral solution at 100° C. and the haptene shows marked resistance to heating both in acid and alkaline solution. There is no relation between rugose-non-rugose variation and S-R- ρ change.

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A HEAT LABILE SOMATIC PROTEIN ANTIGEN (H L S P)
OF VIBRIOS

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Young mass cultures of vibrios, the growth from 5 or 10 sq ft of agar surface at each preparation were beaten to a cream with saline. A few c.c. of chloroform were stirred in, causing the mixture to swell and rendering it slimy. (The ability of vibrios to absorb chloroform as compared with that of other organisms tested is remarkable.) The vibrios were again dispersed in 400-800 c.c. of saline and allowed to stand at room temperature. By sedimentation, expedited by the weight of absorbed chloroform, or centrifugation the saline extract was separated after 24-48 hours from the vibrios, which were extracted a second and a third time with saline.

The clarified saline extracts, usually pulp filtered, were half saturated with ammonium sulphate and the precipitate, formed as a rule mainly of flagella but containing other serologically active substances, was removed by centrifugation and careful filtration through pulp. The filtrate was fully saturated with ammonium sulphate and the abundant precipitate collected by centrifugation or filtration and dissolved in water. The solution was acidified with acetic acid to the approximate limit of immediate precipitation and the acid precipitate—H L S P, the fraction at issue—redissolved in water by careful addition of NaOH to neutrality.

The solution was used for test purposes and for the immunisation of rabbits.

Only about one third of the animals inoculated developed precipitins for H L S P during one month's treatment. Those which did responded vigorously and potent precipitating sera were obtained against several typical cholera and El Tor strains and several other vibrio types. In quantitative tests precipitation was observed with dilutions of the fraction up to 1:250,000.

The antisera against extracts of S cultures sometimes contained smooth specific O antibody and traces of S specific substances could be detected in the extracts themselves. The antisera to preparations from R and ρ cultures were, of course, free from this impurity and were never found to contain the characteristic agglutinating antibodies of ordinary R and ρ antisera; they were therefore particularly useful in studying the essential reaction of H L S P.

Most of the antisera prepared when tested by the method of Vassiliadis (1937) were found devoid of flagellar agglutinins. Indeed there is as yet no evidence that the essential H L S P antibodies cause clumping of vibrio cultures in any of their known variant states at or above dilutions of 1:100.

Solutions of H L S P from S, R and ρ 1 cholerae and El Tor vibrios precipitated indiscriminately and heavily with any H L S P sera. It is clear that the fraction is common to all these vibrios and variants and it is not modified in the rugose culture.

But what is remarkable is that the H L S P cholera antisera reacted equally or almost equally with the corresponding extracts of all or most accredited vibrios. The latitude of the reaction far transcended that of the vibrio flagella and their antibodies. Saline extracts of the vibrio of Tinkler and Prior, an organism without agglutinative similarity, somatic or flagellar,

to *V. cholerae*, precipitated intensely with anti-H.L.S.P. cholera sera. Up to the present only two alleged vibrios—the fluorescent *V. neocistes* (N.C.T.C. no. 2582) and the problematic culture “Rangoon rough 2” (Linton *et al.*, see references given by White, 1940)—have failed, among 58 strains representing some 30 smooth serological types of vibrio, to yield extracts responding vigorously to cholera and El Tor H.L.S.P. antisera. Extracts of the two strains named, of the coliform, *Salmonella*, dysentery, *Proteus*, Morgan no. 1, and *Pseudomonas* groups and of *Spirillum rubrum* gave entirely negative reactions.

While the range of observations is not yet sufficiently wide for confident generalisation, it is possible that a basis exists here for a very wide grouping of vibrios, perhaps even for a serological definition of the genus.

I have advanced (White, 1937, 1940) various reasons for rejecting the culture “Rangoon rough 2,” which has played a special part in the studies and views of Linton and his co-workers, from consideration as a variant of *V. cholerae*. The present observations, coupled with the morphological peculiarity of the organism, raise the question as to whether it is a vibrio at all. It is assuredly no near relative of *V. cholerae*, all witness to the contrary notwithstanding.

The fraction H.L.S.P., so far as has been determined, is precipitable by saturation but not by half saturation with ammonium sulphate, is rapidly coagulated and antigenically inactivated by heating at 100° C. in neutral solution, gives intense positive biuret and Millon reactions and a definite Molisch test, is antigenically and serologically inactivated by digestion with trypsin and papain and by *N*/100 NaOH and is precipitated by acid with gradual coagulation, the optimal reaction being about pH 5.

The characteristic precipitins of the H.L.S.P. fraction commonly occur in the antisera of S, R and ρ vibrios which have not been exposed to destructive heat.

The failure of H.L.S.P. antisera to cause agglutination of vibrio suspensions suggests that the fraction is either a constituent of the deeper bacterial substance, not included in the reactive surface, or that it is a soluble secretion. The fact that relatively little was found to be present in rapid saline washings of young living cultures and that in repeated extractions by the method given successive potent extracts were obtained, inclines me decisively to the first view. The bulk of the fraction itself indicates a somatic rather than a flagellar origin, and the flagellar fraction, precipitated at half saturation with ammonium sulphate and returned to water, shows no steady effusion of H.L.S.P.

Summary

A method is described for separating from vibrios a heat-coagulable protein antigen, common to all known variant forms and seemingly derived from the deeper somatic substance, which, though taking no evident part in vibrio agglutination, shows extremely wide cross precipitation reactions throughout, but not over-stepping, the vibrio group.

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576 851 5 (*Cl novyi*) 576.807A SIMPLE AND RAPID METHOD OF DISTINGUISHING *CL NOVI* (*B. ŒDEMATIENS*) FROM OTHER BACTERIA ASSOCIATED WITH GAS GANGRENE

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(PLATE XXII)

Penfold (1922) originally pointed out the value of compounds of the benzidine series for differentiating cultures of various bacteria on blood agar and subsequently McLeod and Gordon (1922 *a* and *b*) showed that hydrogen peroxide was formed in the cultures of such bacteria as gave the most marked blackening of blood benzidine media, notably the pneumococcus and some streptococci. A good deal later it was shown by McLeod (1930) that there were a few anaerobes which, when grown on the surface of heated blood agar, developed faint green haloes around their colonies about half an hour after the removal of cultures from the anaerobic jar and exposure to air. When such bacteria were grown on heated blood agar containing benzidine, the colonies blackened after exposure to air, the speed of development and spread of the green and black discolourations being very similar. This was advanced as additional evidence for the theory, already supported by other and less direct observations, that the intolerance of anaerobes for oxygen was due to the formation of traces of peroxide.

The theoretical bearing of our observations on the problem of hydrogen peroxide formation by anaerobic bacteria will not be discussed in this paper as its purpose is to draw attention to the fact, till now we believe overlooked, that colonies of *Cl novyi* (*B. Œdematiens*) are capable of producing a green halo on chocolate agar and blackening on benzidine blood agar. The development of a green colour around the colonies of one strain was first observed a couple of months ago when this organism was being subcultured with a view to some observations on the bactericidal power of sulphanilamide. As soon as this had been observed, the effect of adding benzidine to the medium was investigated.

Fig 1 illustrates the appearance shown when a pure culture of *Cl novyi* is grown on benzidine "chocolate" agar under anaerobic conditions for 24 hours and then exposed for 30 minutes or longer to air. The medium used consisted of 1 c.c. of benzidine solution (benzidine 0.25 g., N HCl 0.3 c.c., distilled water 50 c.c.) and 11 c.c. of heated blood agar (i.e. 10 per cent defibrinated rabbit blood in agar heated for 15 minutes at 75° C). The mixture was poured into small plates immediately after the addition of the benzidine solution.

Fig 2 shows the contrast in appearance between the colonies of *Cl septicum* (*vibrio septique*) and those of *Cl novyi* when grown on this medium in mixed culture.

Figs 3 and 4 show similar mixed cultures of *Cl welchii* and *Cl novyi*, fig 3 showing the strong reduction effect observed immediately after opening the anaerobic jar and fig 4 the subsequent differentiation due to the blackening by peroxide of the benzidine around the colonies of *Cl novyi*. This blackening is developed most rapidly if the culture is kept at 37° C after exposure to air rather than at room temperature.

The value of this observation might obviously be considerable in making an overnight differentiation of *Cl. novyi* from other anaerobes in cultures from war wounds and so facilitating the use of specific antiserum at the earliest possible moment. The method is obviously simpler, quicker and less expensive than the inoculation of serum-protected animals with mixed cultures. To assess the possible value of such a test, however, it was necessary to make sure that it was not a feature peculiar to one strain. Through the kindness of Dr H. Henry and Dr R. St John-Brooks we obtained possession of eleven strains of *Cl. novyi*, seven from Dr Henry and four from Dr St John-Brooks. Strains Domange and Jolly were included in both collections. All of the eleven strains, which probably represented at least nine of separate origin, gave colonies of similar appearance and all blackened benzidine "chocolate" agar.

In addition to *Cl. welchii* and *Cl. septicum* (vibrion septique), as shown on figs. 2 and 4, cultures of *Cl. tetani*, *Cl. sporogenes*, *Cl. histolyticum* and a strain of a bacillus with terminal spores of the type of *Cl. tertium* which had been isolated from human faeces gave negative results. On the other hand *Cl. botulinum* and some anaerobic streptococci were found by McLeod in previous work to give slightly positive results.

The practical value of a test of this kind would obviously depend in part on the extent to which anaerobes giving a similar reaction might appear casually in wound discharges owing to their presence in faeces or soil. With a view to assessing the importance of this possible source of confusion, we have examined 20 samples of soil from various points in Leeds or its vicinity, 20 samples of human faeces and 8 of sheep faeces. These were suspended in distilled water and the suspension heated at 70° C. for 30 minutes prior to inoculation on to benzidine "chocolate" agar and incubation under anaerobic conditions. Unheated suspensions of soil were also so inoculated. In all these samples three only gave rise to black colonies. Two of these came from human faeces and one from soil, and all proved to be streptococci capable of growing freely under aerobic conditions.

Summary

Heated blood agar containing benzidine is a suitable medium for the rapid identification and isolation of *Cl. novyi* (*B. œdematians*) and will probably be used with advantage in the study of the anaerobic flora associated with gas gangrene.

We are indebted to Messrs R. Burrow and A. Sturgess for much keen technical assistance and have pleasure in expressing thanks to the Medical Research Council for a grant for expenses.

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ANAEROBES ON HEATED BLOOD AGAR CONTAINING BENZIDINE

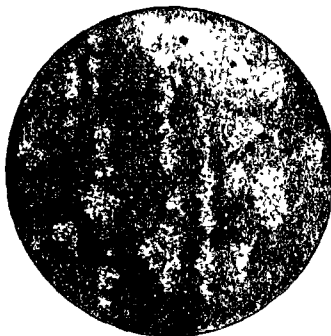


FIG. 2.—Mixed culture of *Cl. septicum* (*vibrio septique*) and *Cl. novyi*. Medium and conditions as in fig. 1.

FIG. 1.—Pure and relatively abundant culture of *Cl. novyi* (*B. oedematiens*) on heated blood agar containing benzidine. A 24-48-hour culture one hour after exposure to air.



FIG. 3.—Mixed culture of *Cl. welchii* and *Cl. novyi* immediately after removal from anaerobic jar. Reduction characteristic of an anaerobe growing on heated blood agar (? hemochromogen formation) is well marked and masks the difference in appearance between the colonies of the two organisms. Medium as in fig. 1.

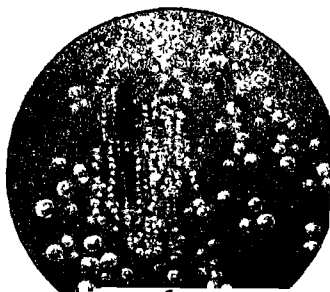


FIG. 4.—Plate similar to that in fig. 3 after exposure to air for an hour or more. The slight difference in the appearance of the colonies observed in the absence of benzidine is much reinforced by the blackening of the colonies.

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COMPLEMENT FIXATION WITH THE SHOPE RABBIT
PAPILLOMA VIRUS

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Kidd (1938) described a complement fixation reaction between the sera of rabbits bearing the Shope papilloma and an extract of the tumour as antigen. He brought forward evidence to show that the reaction was specific and that the effective antigen in the tumour extract was the virus particle itself, there being no evidence of the production of a soluble antigen by the virus such as occurs in vaccinia (Craigie and Wishart, 1936), psittacosis (Bedson, 1936) influenza (Fairbrother and Hoyle, 1937a) and other virus diseases. The object of this investigation was to verify the work of Kidd, especially with reference to the question of the presence or absence of a soluble antigen.

The technique employed in the complement fixation tests was almost exactly that used by Kidd, except that in the author's test a somewhat larger dose of complement was used (2½ as against 2 M H D).

Complement fixing antibody was found to be present in the sera of 16 out of 26 domestic rabbits bearing Shope papillomata, the titres ranging from 1/2 to 1/16, and no such antibody was found in the sera of 20 normal rabbits or in the sera of 10 rabbits bearing the Brown Pearce carcinoma. In 8 rabbits complement fixation tests were done before and at varying intervals after inoculation of virus and it was found that antibody made its appearance in the serum shortly after the first appearance of papillomata and increased in amount as the papillomata enlarged.

Complement fixing antigen was found to be present in various samples of glycerinated cottontail papillomata sent to the author by the courtesy of Dr Kidd and Dr Shope, the amount of antigen being greater in samples which were highly infective than in those of lower infectivity. No antigen could be detected in papillomata of the domestic rabbit and extracts of such papillomata were found to be non infective.

The possible presence of a soluble antigen was tested in the following way. A 10 per cent suspension of glycerinated cottontail papillomata was prepared by the method described by Kidd and samples were filtered through a series of gradocol membranes of different pore size. The various filtrates were then tested for complement fixing antigen and for virus by inoculation into the scarified skin of domestic rabbits.

It was found that gradocol membranes of average pore diameter 400 $\mu\mu$ passed both virus and complement fixing antigen freely, membranes of A.P.D. 200 $\mu\mu$ passed a very small amount of virus and a trace of complement fixing antigen, while membranes of A.P.D. 100 $\mu\mu$ retained both virus and antigen. The particle size of both virus and complement fixing antigen appeared therefore to be the same.

A second attempt to separate the virus and complement fixing antigen was made by a method which had proved successful in the case of the influenza virus (Fairbrother and Hoyle, 1937b). This consisted in the precipitation of the complement fixing antigen from the crude virus suspension by the addition of 0.03 per cent of acetic acid followed by redissolving the precipitate in a small volume of phosphate buffer of pH 7.4. In the case of the influenza virus this resulted in the production of a concentrated

solution of complement-fixing antigen free from virus, the latter being removed during the process of centrifuging out material insoluble in the phosphate buffer. With the Shope papilloma virus this procedure failed, no concentration of complement-fixing antigen being possible, and although the final solution contained a small amount of antigen it was also slightly infective.

From these experiments and those described by Kidd it seems evident that the Shope rabbit papilloma virus does not elaborate a soluble antigen such as occurs in the case of many of the larger viruses, and that the antigen involved in the rabbit papilloma complement fixation reaction is the virus particle itself.

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576 . 851 . 2 (*Streptococcus haemolyticus*) : 576 . 809 . 729

PREPARATION OF HÆMOLYTIC STREPTOCOCCAL ENDOTOXIN

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A rapid method for the bulk production of hæmolytic streptococcal endotoxin is the following. One-litre bottles of horse-muscle digest broth (O'Meara, 1934) are each seeded with 1 c.c. of a 6-hour broth culture of *Streptococcus haemolyticus*. After 18 hours at 37° C. the culture is centrifuged and the deposit washed at least four times in normal saline. After the final washing the organisms are suspended in 100 c.c. of absolute alcohol and shaken at frequent intervals for 2 hours. This last stage is repeated with fresh absolute alcohol for a further 2 hours. The alcohol is then replaced with 100 c.c. of ether and the mixture again shaken for 2 hours. After centrifugation the ether is removed and the defatted organisms are dried *in vacuo*. In this form it has been found possible to store cultures for endotoxin production for at least 2 years at 4° C. When required, 2 g. of dried bacteria are placed in a steel ball mill (Green, 1939) which is enclosed in a jacket packed with solid carbon dioxide at -70° C. Grinding is continued for 3 hours. The mill is then allowed to return to room temperature and its contents are taken up in successive 100 c.c. volumes of buffer solution at pH 8 until a total volume of 1000 c.c. has been used, the constitution of the buffer solution being as follows :—

0.2 M KH ₂ PO ₄	50 c.c.
0.2 N NaOH	46.8 "
Distilled water	to 200 "

After 6 hours' extraction at 6° C the suspension is centrifuged for 1 hour at 3000 *r p m*. The opalescent supernatant fluid is separated and its reaction adjusted to pH 4 by the addition of concentrated HCl. At this stage a white flocculent precipitate appears. This is removed by centrifugation at 3000 *r p m* for 20 min and dried *in vacuo*. The resulting dried white powder is stored as crude endotoxin and is 95.98 per cent protein.

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A COMPARISON OF THE EFFICIENCY OF CERTAIN DRUGS AND DYES IN KILLING CULTURES OF *LEISHMANIA TROPICA*

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Whereas the bactericidal and bacteriostatic effect of drugs and dyes has been extensively investigated, little experimental work has as yet been done on the effect of these substances on *Leishmania*.

Kligler (1924) observed that a 1:20,000 dilution of tartar emetic and of salvarsan killed cultures of *Leishmania* after two weeks. Das Gupta and Dikshit (1929) found that 1:80,000 berberine sulphate inhibited the growth of *Leishmania tropica*, but not 1:100,000. Emetine hydrochloride inhibited in 1:1000 dilution, while 1:10,000 emetine hydrochloride, 1:1000 stibosan and 1:1000 quinine dihydrochloride did not inhibit growth.

The object of the present study was to estimate the relative efficiency of various drugs and dyes in killing cultures of *Leishmania tropica*.

Material and methods

The locally isolated "Nahid" strain of *Leishmania tropica* was used. It was grown on our medium (Senekji, 1939) in 8 oz. "medical flats" with screw caps. The cultures were in their eighth, ninth and tenth generations and were 10-12 days old. They were washed off with and diluted in normal saline until the number of actively motile leptomonads per c.c. was 10,000,000, as ascertained by counting in the blood counting chamber.

The required dilutions of the various drugs and dyes were prepared in sterile distilled water and to 4.8 c.c. of each was added 0.2 c.c. of the standardised fresh leptomonad suspension.

The tubes containing the mixtures were kept at 20° C. and at intervals of 5, 10, 15 and 45 minutes slopes of our *Leishmania* medium were inoculated from them by means of sterile Pasteur pipettes. These cultures were incubated at 22° C. for 7 days and then examined for living leptomonads. Immediately after mixing hanging drop preparations were also made for the direct observation of lysis or inhibition of motility.

In all these experiments special care was directed to sterility in view of the known inhibitory effect of contaminants on the growth of *Leishmania*. They were carried out at a time when the temperature of the laboratory did not exceed 25° C. It is our experience that temperatures higher than this reduce the viability of the organism.

Results

The drugs tested and the results obtained are given in table I.

The results obtained with dyes manufactured by the British Drug Houses Ltd. are given in table II, p. 174.

The action of various drugs and dyes in the presence and in the absence of 5 per cent. normal horse serum is recorded in tables III and IV, pp. 175-6.

Discussion

We are fully aware that the results of *in-vitro* tests of drugs are not necessarily paralleled by their action *in vivo*. Of the drugs which already have a reputation *in vivo*, berberine sulphate and emetine hydrochloride emerge from our tests creditably but not brilliantly. Far more potent *in vitro* are trypanflavine, euflavine and rivanol. Tests in the presence of 5 per cent. horse serum show that the potency of many drugs is slightly lowered, but not that of trypanflavine, rivanol and emetine hydrochloride. Euflavine was not tested, but in view of its chemical relationship to trypanflavine and of the similarity of its action as shown in table I, it would appear likely that this substance also would not have its leishmanicidal properties lowered by horse serum. Horse serum slightly increases the potency of the dyes.

We would suggest that these drugs deserve a trial in the treatment of visceral and cutaneous leishmania infections. Our experiments were carried out with *Leishmania tropica*, but possibly similar results would be obtained with *Leishmania donovani*.

Summary

A method is described for the estimation of the leishmanicidal action of drugs and dyes *in vitro* and the results of experiments with various compounds are recorded.

The most active substances in these experiments were trypanflavine, euflavine, rivanol and the green and violet dyes. It is suggested that clinical trial should be made of these in the treatment of leishmaniasis.

I am indebted to Dr Ibrahim Bey Akif Al-Allusie, Director-General of Health of Iraq, for permission to publish these findings.

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TABLE I.—*Effect of various drugs on Leishmania tropica in vitro*

Drug	Dilution	Appearances in hanging drop	Growth on culture media after exposure to drugs for			
			5 mins	10 mins	15 mins	45 mins
Emetine HCl (Parke Davis & Co)	1:100	Complete lysis	—	—	—	—
	1:1000	" "	—	—	—	—
	1:10,000	Non motile in 5 mins	+	—	—	—
	1:12,500	" " 10 "	+	+	—	—
	1:15,000	" " 15 "	+	+	+	—
Solusalt arsan (Bayer)	1:100	Complete lysis	—	—	—	—
	1:1000	" "	—	—	—	—
	1:10,000	Degenerate forms	++	+	+	—
Mopharside (Parke Davis & Co)	1:1000	Partial lysis	—	—	—	—
	1:10,000	Non-motile in 10 mins	+	+	—	—
Rivanol (Bayer)	1:100	Degenerate forms	—	—	—	—
	1:1000	" "	—	—	—	—
	1:10,000	Non-motile in 5 mins	+	—	—	—
	1:20,000	" " 15 "	+++	+++	+	—
Trypaflavine (Bayer)	1:100	Complete lysis	—	—	—	—
	1:1000	" "	—	—	—	—
	1:10,000	" "	—	—	—	—
	1:20,000	Non motile in 5 mins	++	—	—	—
Euflavine (British Drug Houses)	1:10,000	Complete lysis	—	—	—	—
	1:20,000	Non-motile in 5 mins	+	—	—	—
Atebrine (Bayer)	1:100	Lysis	—	—	—	—
	1:1000	" "	—	—	—	—
	1:10,000	Non-motile in 5 mins	+	+	—	—
Plasmochine (Bayer)	1:100	Degenerate forms	—	—	—	—
	1:1000	" "	—	—	—	—
	1:10,000	Non-motile in 10 mins	+	+	—	—
Salyrgan (Bayer)	1:100	Actively motile	++++	+++	++	+
Cyanide of mercury	1:1000	Degenerate forms	—	—	—	—
	1:5000	" "	—	—	—	—
	1:10,000	Motile	++	+	+	+
Prontosil sol (Bayer)	1:100	Actively motile	++++	++++	++++	++++
May & Baker 693 (Dagenan)	1:100	Motile	++	+	+	+
Uleron (Bayer)	1:100	"	+++	+	—	—
Albucid (Schering)	1:100	"	+	—	—	—
Anthiomaline (May & Baker)	1:100	Actively motile	++++	++++	++++	++++
Fouadine (Bayer)	1:100	" "	++++	++++	++++	++++
Berberine sulphate (Burroughs Wellcome & Co)	1:100	Complete lysis	—	—	—	—
	1:1000	" "	—	—	—	—
	1:10,000	Non-motile in 5 mins.	+	+	—	—

In all tables — = no growth in culture tubes, + to ++++ = varying amounts of growth in culture tubes

TABLE II.—*Effect of various dyes on Leishmania tropica*

Dyes	Dilution	Appearances in hanging drop	Growth on culture medium after exposure to dyes for			
			5 mins.	10 mins.	15 mins.	45 mins.
Neutral red . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	Degenerate forms	—	—	—	—
	1 : 100,000	Non-motile in 10 mins.	+++	+++	—	—
Methyl red . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	Degenerate forms	—	—	—	—
	1 : 100,000	Non-motile in 5 mins.	+	+	—	—
Fuchsin . . .	1 : 500	Degenerate forms	—	—	—	—
	1 : 1000	Non-motile in 15 mins.	++	+	+	—
Scarlet red . . .	1 : 1000	Non-motile in 5 mins.	++	+	—	—
Safranin . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	Degenerate forms	—	—	—	—
	1 : 50,000	" "	—	—	—	—
	1 : 100,000	Motile	++++	++++	++	+
Chrysoidine . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	Degenerate forms	—	—	—	—
	1 : 100,000	Non-motile in 10 mins.	++++	++	—	—
Orange G . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	Degenerate forms	—	—	—	—
	1 : 100,000	Non-motile in 10 mins.	+++	++	—	—
Methyl violet . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	Degenerate forms	—	—	—	—
	1 : 50,000	" "	—	—	—	—
	1 : 100,000	" "	+	—	—	—
Crystal violet . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	" "	—	—	—	—
	1 : 50,000	Degenerate forms	—	—	—	—
	1 : 100,000	" "	+	—	—	—
Hæmatoxylin . . .	1 : 1000	Non-motile in 15 mins.	++	+	+	—
Thionine blue . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	" "	—	—	—	—
	1 : 50,000	Degenerate forms	+	—	—	—
	1 : 100,000	Motile	++++	++	+	+
Toluidine blue . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	Degenerate forms	—	—	—	—
	1 : 50,000	" "	—	—	—	—
	1 : 100,000	Motile	++++	+++	+	—
Aniline blue . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	Degenerate forms	—	—	—	—
	1 : 100,000	Non-motile in 10 mins.	++	+	—	—
Brilliant green . . .	1 : 1000	Complete lysis	—	—	—	—
	1 : 10,000	Lysis	—	—	—	—
	1 : 100,000	Degenerate forms	(only 1)	—	—	—
	1 : 200,000	Non-motile in 5 mins.	++	+	—	—
Malachite green . . .	1 : 100,000	Degenerate forms	—	—	—	—
	1 : 200,000	Non-motile in 5 mins.	+	+	—	—
Methylene blue . . .	1 : 1000	Lysis	—	—	—	—
	1 : 10,000	Degenerate forms	—	—	—	—
	1 : 50,000	" "	—	—	—	—
	1 : 100,000	Non-motile in 15 mins.	++	+	+	—

TABLE III

Growth of *L. tropica* after exposure to various drugs in presence and in absence of serum

Drugs	Dilution	Exposed with 5 per cent horse serum for				Exposed with no horse serum for			
		5 mins	10 mins	15 mins	45 mins	5 mins	10 mins	15 mins	45 mins
Rivanol	1 10,000	++	-	-	-	+	-	-	-
Trypaflavine	1 10,000	1 only	-	-	-	-	-	-	-
Atebrine	1 10,000	+	+	-	-	+	+	-	-
Plasmochino	1 10,000	++	++	+	-	+	+	-	-
Solusaltarsan	1 10,000	++++	++	+	-	+	+	+	-
Mepharside	1 10,000	++	+	-	-	+	+	-	-
Emetine HCl	1 10,000	+	-	-	-	+	-	-	-
Berberine SO ₄	1 10 000	+	+	-	-	+	+	-	-
HgCN	1 10 000	++++	++	+	+	++	++	+	+
Salyrgan	1 100	++	+	+	+	++	++	++	+
Anthomaline	1 100	++	++	++	+	++	++	+	+
Foundine	1 100	++	++	++	+	++	++	+	+
Prontosil sol	1 100	++	++	++	++	++	++	++	++
Dagonan (M & B 693)	1 100	++	++	++	++	++	++	+	+
Ulcron	1 100	++	++	++	++	++	+	-	-

TABLE IV

Growth of L. tropica after exposure to various dyes in presence and in absence of serum

Dyes	Dilution	Exposed with 5 per cent. horse serum for				Exposed with no horse serum for			
		5 mins.	10 mins.	15 mins.	45 mins.	5 mins.	10 mins.	15 mins.	45 mins.
Neutral red	1:100,000	+	-	-	-	++	++	-	-
Methyl red	1:100,000	+	-	-	-	+	+	-	-
Fuchsin	1:1000	++	+	+	+	++	++	+	+
Scarlet red	1:1000	+	+	-	-	++	++	-	-
Chrysoidine	1:100,000	+	+	-	-	++	++	-	-
Orange G	1:100,000	+	+	-	-	++	++	-	-
Methyl violet	1:100,000	+	-	-	-	+	-	-	-
Crystal violet	1:100,000	+	-	-	-	+	-	-	-
Aniline blue	1:100,000	+	+	-	-	++	++	-	-
Toluidine blue	1:100,000	++	-	-	-	++	++	+	+
Methylene blue	1:100,000	++	+	-	-	++	++	+	+
Haematoxylin	1:1000	+	+	-	-	++	++	+	+
Brilliant green	1:200,000	+	+	-	-	+	+	-	-
Malachite green	1:200,000	+	+	-	-	+	+	-	-



W. H. Henshaw

OBITUARY NOTICES OF DECEASED MEMBERS

William Howel Evans

1891-1939

(PLATE XXIII)

WILLIAM HOWEL EVANS was born in Liverpool in 1891 and in that city he lived and worked until his death in his 48th year. He received his schooling in the Liverpool College and in after life he showed in many ways his appreciation of the educational benefits he had received from that institution. A row of school-crested, leather-bound volumes on one of his bookshelves gave evidence of a schoolboy brilliance such as those who met him later would have expected.

He matriculated in October 1909 and entered the Faculty of Medicine in the University of Liverpool. His first four years were devoted to pure science for, instead of proceeding with the normal medical course after passing his first M.B., Ch.B. examination, he followed his keen interest in chemistry and in 1913 took an honours B.Sc. degree in biochemistry. During the last year of these studies he worked, under Benjamin Moore, on the problem of polyneuritis in pigeons and in particular on the search for the anti-neuritic factor now known as vitamin B₁. In the session 1913-14 he resumed his medical studies and with the outbreak of war, like so many others, continued just so far along his course as was demanded of him before going on active service. In 1916 he joined the R.N.V.R. as surgeon-probationer and served in various theatres of war, but chiefly as surgeon sub-lieutenant on the escort sloop *Cornflower*. Early in 1918 he was released from the navy to complete his medical course and in March 1920 he qualified with second class honours.

After a term as house-physician to Dr John Hay, during which he had recurring attacks of deafness, he was appointed, in October 1920, Holt fellow in pathology and worked for twelve months under Professor Ernest Glynn. In 1921 he returned to the Royal Infirmary as medical registrar and tutor and held this post for fifteen months. In later years he always appeared to look back on his medical tutorship as one of the happiest periods of his life.

Admirably fitted for the work both by reason of a real aptitude for teaching and by a thorough grounding in the basic studies of biochemistry and pathology, he was an outstanding success as a tutor. He was a hard task-master to his clerks, but all who passed through his hands speak ungrudgingly of the painstaking care he bestowed on patients and students alike. His case sheets were models of completeness and meticulous tidiness, and whenever possible he enriched them with his neat clear pen-and-ink illustrations of the lesions diagnosed. It was thus a saddening blow to him when renewed attacks of deafness and the unhopeful verdict of an eminent aurist forced him to recognise the fact that consulting medicine was not for him. Without hesitation he decided to devote himself to pathology and in January 1923 he returned to Professor Glynn's department. He completed the rest of this session as Holt fellow and in October he was appointed lecturer in pathology.

For the next six years he was Glynn's right hand man, sharing in all the laboratory and academic undertakings of that enthusiastic and energetic worker. Evans was the only full-time member of Glynn's staff during this period and on him devolved all the time-absorbing duties of providing for the practical classes as well as much of the lecturing to dental and veterinary students for whom classes were run in addition to the routine medical courses. In spite of this he found time for a considerable amount of original work and his own researches supplemented the investigations of the Holt fellows of those years, as his publications bear witness.

His interest was naturally in the pathological investigation of clinical conditions and all his published papers show evidence of exacting biochemical and hæmatological studies, often combined with the most careful histology. Hæmatology was his major interest and during these years he laid the foundations of that expert knowledge in this branch of medicine which was later to establish him as one of the most trusted specialists in blood diseases in the North of England. In addition to his classical exposition of the relation of platelets to thrombosis in post-operative and especially in post-splenectomy cases and in Banti's disease, his investigations included studies of the leukæmias, purpura and hæmophilia. In all his work he was never satisfied with anything which did not bear the hall mark of real originality. This was no mere intellectual snobbishness nor an intolerant decrying of the value of confirmatory work, but simply a refusal, so far as he was concerned, to publish anything which did not add something new to the sum of knowledge of the subject treated.

In 1927 the serious illness of his chief added greatly to his departmental duties. From the time that Professor Glynn was

taken ill until the appointment of Professor J H Dible to the chair in 1929, Evans was in charge of the department and, with only part time assistance from a few clinicians, he carried the responsibilities single handed. It was strenuous work and a trying time, but he stood up to it cheerfully, and the students of that period retain the happiest recollections of his animated and stimulating lecture discussions. Many of them who have since attained senior clinical and academic rank maintain that it was in these classes they first learnt the value and interest accruing from a personal resort to original papers as a logical extension of their text book studies.

Amongst other changes which followed Glynn's death was the decision by the Royal Infirmary to appoint a clinical pathologist in sole charge of their routine pathological work. Evans was asked to accept this appointment and, though reluctant to sever his direct connection with academic pathology and in particular his happy contact with students, he decided to undertake the work. From October 1929 until his death he was in charge of this clinical pathological laboratory housed in the basement of the University pathological department and here he established a high tradition of efficient and disinterested service. His motto was "prompt reports", but he never allowed this to cloak slipshod or uncompleted work, and he fought hard to raise the standard of clinical pathology. He was particularly insistent on the cultivation of a spirit of co operation between clinician and pathologist, refusing to condone any tendency on the part of clinical colleagues to regard his work as ending with the bare record of a chemical estimation or a bacteriological finding. Wherever possible he visited the patient and his report related his findings to the clinical condition exhibited.

During these nine and a half years he built up an extensive private practice. Work came to him from all parts of Merseyside and often from further afield, especially from North Wales. His medical colleagues paid him the high compliment of seeking his assistance, both in diagnosis and treatment, whenever they themselves or their families required medical aid. His help was particularly sought in controlling the treatment of diabetes and pernicious anaemia, and many sufferers from these diseases attended his consulting rooms in Rodney Street, often as non paying patients. In addition to the work at the Liverpool Royal Infirmary and his private practice he was responsible for the pathological work of the St Paul's Eye Hospital. All this meant long hours of work, and the light in the laboratory at 36 Rodney Street was only too often to be seen burning in the small hours of the morning. He was consideration itself to his technical assistants but he did not spare himself, he seldom took enough holiday and this undoubtedly

sapped his strength and contributed in no small measure to his early decease.

Evans joined the Pathological Society in 1924 and two of his most important contributions were published in this *Journal*.^{*} In 1926 he was appointed secretary to the joint committee of the Medical Research Council and the Pathological Society which was set up to investigate the problem of status lymphaticus. He took a large share in the organisation of this work and was responsible for collecting and collating the information from the various investigators up and down the country. These data formed the basis of the report presented in 1931 by Young and Turnbull (this *Journal*, xxxiv, 213). For reasons already given his earlier regular attendance at the meetings of the Society gradually gave way to infrequent appearances. The last time he was present was at the summer meeting in Liverpool in 1935. It was so too with his connection with the Liverpool Medical Institution, which he joined in 1920 and in which he held office as secretary of the pathological section in 1928-29. Latterly he was rarely seen at the meetings though he always inquired closely into the papers and exhibits from any of his colleagues who had attended the meetings.

From 1929 onwards he held the appointment of honorary lecturer in clinical pathology and in June of that year he was awarded the M.D. of the University, having submitted as his thesis his collected papers on the blood platelets. His interest in hæmatology found expression in another direction. He was largely responsible for the establishment of the Merseyside blood transfusion service, and in the beginning he was mainly responsible for its policy. Even after he had left the University department he continued this work and, with the professor of pathology, eventually undertook all the group-testing of donors.

As a young man he took a keen interest in outdoor sports. At "rugger" he was an extremely nippy scrum half and he was no mean hand at cricket; but it was at hockey that he showed to best advantage. He represented his University and got his county cap, playing in the Lancashire team which toured the southern counties in 1922. In later life his chief interest was cricket and he rarely missed a good county match. His knowledge of the finer points of the game was that of an expert and he proudly fostered the growing interest and prowess of his young son in this direction. His other interests were to some extent influenced by his deafness. His gradual withdrawal from participation in the activities of the

^{*} "The blood changes after splenectomy in splenic anæmia, purpura hæmorrhagica and acholuric jaundice, with special reference to platelets and coagulation" (1928, xxxi, 815), and "The relation of the blood platelets to thrombosis after operation and parturition" (1928, xxxi, 833), the latter jointly with R. Y. Dawbarn and F. Earlam.

various medical societies of which he was a member has already been mentioned. So it was in his social life. At one time a keen student of drama and an inveterate Gilbert and Sullivan fan, he gradually gave up everything which meant going out in the evenings. Reading became his chief pastime. He read widely, with particular selection of historical novels and biographies. Like so many medical colleagues he found considerable pleasure in detective stories and in this field his choice and recommendations were frequently sought by his friends. He was amusedly proud of his detailed knowledge of the technique of Sherlock Holmes and followed with interested delight the pseudo scientific investigations of the Sherlock Holmes Club. His interest in the past is reflected also in his chief hobby—the collection of antique furniture. His home contained many beautiful examples of the finest craftsmanship of the Queen Anne and Chippendale periods though he never indulged in extravagant acquisition of show pieces. Almost every article of furniture in his house represented a personal triumph both of perspicacity in purchase based on expert knowledge and of loving care and devoted attention in the processes of cleaning, polishing and faithful restoration. His appreciation of the beauty of expert craftsmanship explained also two other features obvious to all visitors to his home, namely the modest but carefully selected collections of Lalique glass and of the coloured woodcuts of Urushibara. Just as these artistic tastes exemplified his striving after technical perfection so his other hobby—philately—bore testimony to the importance he attached to detail. He was no mere collector of stamps. His collection was a highly specialised one and aimed at illustrating the technique of production and distribution of the postage stamp.

Despite his heavy duties and time absorbing hobbies and occupations, Howel Evans was always available to all and sundry who sought his companionship, advice or help. He was generous to a fault and never spared himself when he could be of help to anyone. His wide reading and remarkably retentive memory made of him a welcome companion and an intelligent conversationalist. He latterly showed an irritability foreign to his nature, but this, we realised later, was but the inevitable manifestation of his ill health, and it was always obvious that no one regretted this irascibility more than himself. He married in July 1924 Sally Capper who survives him, and he leaves two children, a boy of fourteen years and a girl of eleven. In recent years the "parental" education of these children absorbed his attention to a very great extent and here again the thoroughness which characterised all his work was manifested. He became a mine of information on children's books, and his library of these had become almost an institution among the children of his numerous friends.

The death of Howel Evans was sudden, and is regarded as a catastrophe to the medical world of Liverpool. Men of his intellectual attainments are rare and clinical pathologists of his standing rarer still. His passing has left a gap in the medical consultant ranks of Liverpool which will not easily be filled.

T. B. D.

John Gray

1900-1939

JOHN GRAY, who died in London on 26th August 1939, was, at the time of his death, reader in morbid anatomy at the British Post-graduate Medical School of the University of London. Born in 1900, the son of John and Christina Gray of New York, he was educated at Heriot's School, Edinburgh, which he left in 1918 to serve as a private in the Highland Light Infantry, but on account of his age he did not see foreign service. At the end of the war Gray proceeded to Edinburgh University, having previously gained the Heriot bursary. His career as a medical student was marked by brilliant successes; he being medallist in his year in seven subjects, as well as gaining the Vans Dunlop scholarship in anatomy, the Murchison memorial and Stark scholarships in clinical medicine and, on graduation, the Allan fellowship in clinical medicine and surgery. He took his bachelor's degrees with honours in 1923 and four years later was awarded a gold medal for his M.D. thesis on nephritis.

After graduation Gray worked for a time in the department of anatomy in Edinburgh and held a resident medical appointment in the Royal Infirmary. His heart, however, was towards the scientific side of medicine and early in 1925 he joined the staff of the pathological department of the University of Aberdeen under Theodore Shennan, holding at the same time the Leckie-Mactier post-graduate fellowship of Edinburgh. Almost at once Gray began to publish. His first paper—on polyarteritis nodosa—appeared in this *Journal* in 1926 and from that time up till the appearance of his last paper—on malignant infarcts in the placenta, written with Kenny and Sharpey-Schafer—he continued to turn out sound and scholarly work upon morbid anatomical subjects. Gray's magnum opus, however, and the study by which he is best known and will be chiefly remembered by his colleagues, was his monograph on nephritis, published in 1933 as one of the Special Report Series

(no 178) of the Medical Research Council. Here he revealed his quality, for in tackling a subject of the greatest difficulty he showed an admirable grasp of all its aspects, and by bringing into compact and readable form the clinical, morbid anatomical and functional sides of this difficult subject, he performed a real *tour de force*. The material investigated was all handled by him personally and his review and critical analysis of the problems of nephritis have been of the greatest service to pathologists. In 1934 Gray left Aberdeen for London, taking up the newly established post of director of pathology and Lyle research scholar at Queen Mary's Hospital for the East End, and in the following year he applied for and was appointed to the Postgraduate School readership.

Here Gray entered upon a new phase of his career. He had established himself as one of the best of the younger morbid anatomists and had attained to a senior position which gave him both freedom to organise his work upon lines which he desired personally, and reasonable scope for research. He settled down to the construction of his part of the department which was growing up under Kettle's wise and sympathetic guidance, and was responsible in no small degree for the organisation of the section of morbid anatomy and the building up of the *matériel*—so precious and so difficult to acquire—upon which a well equipped laboratory of morbid anatomy bases itself. The work prospered and the department took shape. Gray's teaching was acceptable and his opinion sought and depended upon. He was experienced, cautious, wise in judgment, and never used dogmatism as a cloak for ignorance. When appealed to for an opinion in a conference he would give it slowly and with an economy of words, but clearly and with the reasons upon which it was based.

Then illness fell upon him. He had never looked robust and for a time he had appeared to be less so than usual. One afternoon, in the autumn of 1937, he came into the writer's room to find him endeavouring to oust an intruding sparrow. Gray assisted in the operation and when it had been successfully completed he told me that he had just learned that he was suffering from pulmonary phthisis. He said that he had lately felt himself lacking in his usual keenness for his work and was relieved to find this physical basis to explain what he had attributed to blameworthy inertia. The School gave him a year's sick leave and at the beginning of the 1938 session he reappeared, apparently in good physical condition, whilst the public and privately expressed opinions of his doctors were encouraging—his lesion was not healed, but it was an old fibrotic one and with light work the prognosis was excellent. Unfortunately, hopes based upon such prognostications proved vain. His disease reappeared in an aggravated form in the spring of the year and gave no hope for his recovery.

John Gray was an example of a man of brilliant parts which his physique was unable to sustain. Without assistance and by his own gifts and unaided efforts he toiled towards the upper ranges of his profession. At last, when the future seemed assured and a chair of pathology a moral certainty,

“ Comes the Blind Fury with th’ abhorred shears
And slits the thin-spun life.”

He was a good and wise colleague.

J. H. D.

BOOKS RECEIVED

Handbook of hematology

Edited by HAL DOWNEY 1938 London Hamish Hamilton Medical Books Four volumes, pp 3136, 1448 figs, including 50 plates in colour £16 16s

A work of this magnitude, magnificence and cost must indeed be intimidating even to the stoutest hearted reviewer. Specialisation in medicine, however, entails either encyclopedic works by groups of specialists of the type now before us or a large number of smaller monographs dealing with separate parts of a subject. The chief merits of the encyclopedic method are convenience and accessibility, the chief disadvantage is the difficulty, nay the virtual impossibility, of keeping the volumes up to date. The length of time required to produce an encyclopedic work means that inevitably some of it is out of date before it is published. In the present instance, neither in the discussion of jaundice nor of hæmorrhagic disease of the new born is there any mention of their relation to prothrombin or of the recent extensive literature dealing with vitamin K, while in all probability the very exhaustive chapter on the mast cell was already in proof by the time (1937) the Scandinavian work on the relation of this cell to heparin had appeared.

In keeping with the character of these volumes this notice is the work of several reviewers. We much regret the delay in its appearance and would offer our apologies to Dr Downey and his collaborators, and to the publishers.

Be it said at the outset that the publication of this handbook fills a long felt need in the field of hæmatology. It is, without question, the most authoritative and comprehensive treatise on this rapidly expanding subject as yet published in any language. "Handbook" would appear to be a misnomer for a work of such encyclopedic dimensions. Indeed many of the contributions are full dress monographs. It is impossible to do justice to such a work without first gaining familiarity through long contact and constant reference. In general, however, we have found the subjects presented in a clear, concise and interesting manner. The inclusion of an extensive bibliography at the end of each section is one of the most valuable features of the work.

This *Handbook* has been made available through the efforts of an able editor, Dr Hal Downey, and thirty seven collaborators, most of whom have themselves made important contributions to the subjects they discuss. With the exception of Meulengracht, who contributes the section on chronic hereditary hemolytic jaundice, and Franz Josef Lang of Innsbruck, who writes on myeloid metaplasia, all the authors are resident in the United States of America. The editor has allowed his collaborators considerable latitude, and in consequence there is a certain amount of repetition. On the other hand the method permits an equitable consideration of widely divergent points of view. It is unfortunate, however, if this policy has been responsible for certain aspects of hæmatology receiving little or no attention. It is disappointing, particularly at the present time, to find the practical aspects of blood

grouping and blood transfusion given such scant attention, while the paroxysmal hæmoglobinurias, so admirably discussed by Witts in 1936, receive no consideration.

As the editor indicates in his preface, the *Handbook* has been designed to give the hæmatologist an adequate knowledge and appreciation of the fundamentals upon which his science is based, as a preparation for the consideration of the hæmatopoietic system in disease. It is therefore not surprising to find over one-half of its pages devoted to the more basic aspects of the subject, but in general the more practical aspects of hæmatology also receive adequate treatment. In our opinion, however, disease is occasionally treated too much from the purely morphological aspect of the blood cell, and the complementary clinical, serological and biochemical data are not always given sufficiently full consideration.

The opening chapter of volume I is a weighty contribution by Raphael Isaacs on the development and morphology and the physical and chemical properties of the erythrocyte. It contains much entertaining information about red cell characters in birds, amphibia and other animals as well as man. The author's own views on the development of red cells appear to agree with those of Turnbull, though Turnbull's work on the marrow in man is not mentioned. The conclusions of the various schools of thought on development are discussed. This section incorporates a digest of an enormous volume of literature and is supplemented by a rich and extremely valuable bibliography. Many subsections, however, lack critical evaluation of the data presented, and it is frequently necessary to return to the original articles for a true perspective.

The sections on the polymorphonuclear neutrophilic leucocytes and on the functions of the leucocytes by Bunting are clear and concise, but didactic in character. Eosinophilic leucocytes and eosinophilia are adequately discussed by Ringoen. Section IV, by Nicholas A. Michels, is a veritable monograph on the mast cell and extends to 142 pages, with a bibliography of over 400 references. There are many histological illustrations and two particularly fine plates in colour. Much detailed information is provided on the comparative anatomy and physiology of the mast cells and it is interesting to read the 16-page account of the various theories of their function in the light of the recent claim by Jorpes, Holmgren and Wilander that this is no less than the formation and secretion of heparin.

Bloom, in the section on lymphocytes and monocytes, presents a detailed exposition of the unitarian theory of blood formation as represented by the Maximow school. The histological and experimental evidence for the extreme monophyletic view of hæmatopoiesis is marshalled in a careful and orderly fashion. The chapter includes valuable schemata of other theories of blood cell formation.

N. Rosenthal's section on blood platelets and megacaryocytes is remarkable, even in such a finely produced book, for its excellent series of microphotographic illustrations.

Three sections are devoted to supravital methods of studying blood cells. Cunningham and Tompkins, who have had an extensive experience in this field, describe their technique in detail and discuss its application to the study of the blood in pathological conditions. We are inclined to agree with Hall in his "Evaluation of supravital staining" that the dry smear method is decidedly superior to the supravital technique as a routine laboratory method.

Rosenthal discusses the hæmorrhagic diatheses in a section devoted

to the purely clinical aspects of these diseases. The numerous clinical classifications which have been evolved, based on complex physiological processes and multiple aetiological factors are of somewhat doubtful value. While Rosenthal includes thrombasthenia in his classification, he submits no evidence for the existence of this supposed defect. The absence of any reference to the recent work on vitamin K and on the relation of prothrombin to hemorrhage in jaundice has already been commented on.

The nine sections of volume II comprise articles of fundamental importance on the comparative anatomy, morphology and physiology of the blood forming organs. H. E. Jordan, in a scholarly contribution on comparative hematology, discusses the blood and hæmatopoietic tissues of fish, reptiles, birds and mammals. The information here assembled and the extensive bibliography will be of permanent value to the comparative anatomist and pathologist. From his comparative studies, Jordan adduces evidence for the totipotentiality of the lymphocyte in hæmatopoiesis. Four sections of this volume are contributed by Bloom, in which he further develops the extreme unitarian view of the Maximow school. His presentation is lucid and his style stimulating. The illustrations in these sections are of exceptionally fine quality. The chapter on tissue culture is interesting but it must be admitted that the results obtained to date by this method have been of minor significance.

An article of monographic proportions by the late R. H. Jaffé of Chicago is devoted to a consideration of the reticulo endothelial system. The presentation is scholarly and systematic, but in striving at completeness the author has discussed many lesions which have no direct bearing on either hematology or the hæmatopoietic system. It is interesting to compare the views of Jaffé with those of Downey who writes the section on monocytic leukemia. Jaffé questions the existence of monocytic leukemia as a separate entity, holding that the blood picture is merely the result of a hyperplasia of the reticulo endothelial system such as may accompany sepsis. Downey, on the other hand, after carefully evaluating the available data and analysing the reported cases, considers it a distinct variety of leukemia. Downey recognises two forms of monocytic leukemia, one in which the monocytes are derived from the reticulo endothelial system (Schilling type), the other where they develop from myeloblasts—in other words a variety of myelogenous leukemia (Naegeli type).

Three sections of volume III are devoted to a study of the bone marrow. Sabin and Miller discuss the normal marrow, Doan its normal and pathological physiology, and O. P. Jones the cytology of the pathological marrow with special reference to biopsy material. This last chapter contains an excellent review of the megaloblast question. While the controversy which has arisen about the use of this term may seem academic or unimportant, such is not the case. Upon its proper use depends the interpretation of the pathology of pernicious anemia and other related conditions. Ehrlich, in 1880, was the first to distinguish two kinds of erythropoiesis, the normal type found after birth (normoblastic) and that present in the foetus or in adults suffering with Addisonian anemia. The greater part of the confusion in English medical literature with regard to the use of the term megaloblast has originated with Doan, Cunningham and Sabin (1925), who redefined the megaloblast as the earliest cell of the erythrocyte series, whether in foetal or in adult life. This is obviously not the immature hemoglobin containing cell described by Ehrlich nor is the view tenable that foetal and adult

erythropoiesis are similar. This misconception is reflected in most of the American hæmatological literature. The majority of British hæmatologists, with the notable exception of Whitby and Britton (1939), do not regard the megaloblast as a normal constituent of adult bone marrow and as a normal precursor of the normoblast. We are in agreement with the views of Naegeli (1931), Ferrata (1933), Jones (1934) and Turnbull (1936) that the megaloblast, as originally described by Ehrlich, is a pathological cell found only in anæmias resulting from a deficiency of the essential "P.A." factor. In many of the sections of the *Handbook*, the term "megaloblast" has been employed without clearly defining what cell is indicated. This can lead only to confusion.

The chapter on the myeloblast by Downey contains an excellent summary of the various theories of hæmatopoiesis and the known facts regarding the relationship of the blood cells. This chapter and the section contributed by Jones, illustrated as they are by exceptionally fine colour plates, give a clear conception of the theory of hæmatopoiesis supported by Downey's unitarian school.

The sections dealing with the anæmias are unquestionably the least satisfactory in the *Handbook*. The discussion on the correct classification of the anæmias is at best confusing. Apart from the common cold, iron deficiency anæmia due to various causes is the most widespread ailment of the human race, yet it has to be content with brief mention in a discussion on classification. The important problem of iron metabolism, on which much evidence has been collected in the last few years, is not even considered. On the other hand 79 pages are devoted to an admirable discussion of a rare condition like polycythæmia. This contribution, by G. A. Harrop and M. M. Wintrobe, is a complete analysis of symptomatic and idiopathic polycythæmia, including both clinical and hæmatological aspects. It should prove a standard reference. The same lack of balance is notable in the treatment of the blood diseases of children. A whole chapter is devoted to a careful analysis of the blood picture of the infectious diseases occurring primarily in childhood, while erythroblastosis foetalis is dismissed in half a page.

In vol. IV the chapter on the pyrrole pigments with particular reference to normal and pathological hæmoglobin metabolism, contributed by C. J. Watson, is an invaluable review of an increasingly important aspect of hæmatology. Much of the literature in the past has been published in scattered German periodicals. Watson has gathered it all together and presented for the first time in English a clear statement of the present position of the whole problem of hæmoglobin metabolism. This review is enriched by much original work. To those unfamiliar with recent advances in the subject some of Watson's statements may appear revolutionary. Those who are already working in this field may not agree with all his conclusions but they will be the first to recognise the value of his facts. The hæmolytic anæmias have long been a waste-paper basket into which any unclassified anæmia was cast. Watson's analysis of hæmoglobin metabolism leaves no excuse for not attempting in any given case to obtain a measure of the degree of hæmolysis occurring. Advance in any subject comes when the power of measurement is available. Watson's account of pigment metabolism in pernicious anæmia and in hereditary hæmolytic jaundice is a necessary complement to the very pedestrian discussions of these conditions provided in other chapters. Sick-cell anæmia to English readers is an academic problem, since it is largely confined to American

negroes, but even English readers will be surprised to find the name of L. W. Diggs omitted from the bibliography.

Infectious mononucleosis is discussed by Heck. The salient features of the disease are admirably documented and are suitably illustrated by case reports from the clinical material of the Mayo Clinic. Downey's excellent description (1923) of the abnormal lymphocytic cells encountered in this disease and his fine coloured illustrations are incorporated. While the experienced hematologist should find no difficulty in distinguishing these 'leucocytoid' lymphocytes from the immature cells of lymphatic leukaemia or from large monocytes, the inclusion of a section on the heterophile antibody reaction will be a boon to many a hospital pathologist. This chapter, contributed by Davidson, gives complete details for the technical performance and interpretation of the test.

A chapter entitled "The action of benzol" but including also the effects of alpha and gamma rays upon the blood and blood forming organs, is written by Selling and Osgood. After a review of a large number of publications on these subjects they conclude with an excellent summary based not only on a critical analysis of the literature but also on their own personal experience. The whole forms a most useful and comprehensive chapter, ranging from toxic actions on the bone marrow and other systems to therapeutic effects and clinical symptoms.

Rosenthal discusses agranulocytosis, a subject which has attracted much attention during the past few years. He emphasises the fact that drugs are responsible for the development of agranulocytosis in the majority of cases and gives a good summary of the literature. No mention is made however of the severe cases which not infrequently follow gold therapy in rheumatoid arthritis, nor is there any statement as to how sulphanilamide and related compounds may affect the blood picture. The descriptions of the morbid anatomy of the disease are clear and concise and are illustrated by a number of exceptionally good microphotographs. Rosenthal is sceptical as to the beneficial results claimed for some of the newer methods of treatment, such as pentose nucleotide.

Richter deals with leucocytosis as well as with the problem of leukaemia. Much of the descriptive material pertaining to this disease lacks conciseness and there is considerable repetition. An interesting subsection describes leukaemia occurring spontaneously in the lower animals. Experimental leukaemia receives careful and discriminating treatment. The discussion of the clinical aspects of human leukaemia however, is less informative. The question of differential diagnosis is inadequately presented and there is no critical evaluation of methods of treatment. Richter's discussion on the nature of the leukaemic process is excellent. He rightly points out that much more information is needed before leukaemia can be ascribed to neoplasia, as has been claimed by a number of leading pathologists in this country. Evidence is lacking to prove conclusively that leukaemic cells consistently present any feature other than immaturity, or that the cell content of leukaemic blood differs in any other way than quantitatively from normal bone marrow. As Richter suggests there is a growing volume of evidence that the maturation of leucocytes is under the control of factors somewhat analogous to those obtaining in pernicious anaemia. A short article on leukaemia in infants and children is contributed by Mettier and Lucas. In our opinion, the use of the peroxidase reaction and of the supravital staining technique does not assist materially in differentiating

a lymphoblast from a myeloblast when the ordinary stained film has failed to yield a clue.

An interesting section on lymphosarcoma and leucosarcoma, including their interrelations with Hodgkin's disease, is written by C. J. Watson. While regarding lymphosarcoma as a true malignant tumour capable of dissemination by lymph or blood vessels and Hodgkin's disease and leukemia as systemic diseases of unknown etiology, the author discusses fully, from the literature and from his own experience, those puzzling cases in which lymphemia may occur as a result of invasion of the blood stream by lymphosarcoma and those other cases of Hodgkin's disease and leukemia which may present nodular lesions of an invasive character. From these transitions and mixtures he concludes that there is a fundamental relationship in this group of diseases. The author rightly deprecates the use of the term "lymphoblastoma" to include Hodgkin's disease, lymphosarcoma and aleukemic leukemia and does not favour the separation from the leukemias of a group of "Leukosarkom" (Sternberg).

The volumes of this *Handbook* are printed on fine quality paper and the illustrations are not only numerous but well chosen and beautifully reproduced. It is unfortunate that in such a monumental work of reference the index should not be fuller: it will be disastrous if the necessarily high price of the work should curtail its use. The *Handbook* should be in every medical library and medical school, for without doubt it will remain the most authoritative treatise on hæmatology for many years to come. The editor, his collaborators and the publishers are deserving of the warmest commendation.

Pathological histology

By ROBERTSON F. OGILVIE. Edinburgh: E. & S. Livingstone. 1940. Pp. x and 332; 220 photomicrographs in colour. 27s. 6d.

This is a book of pictures rather than a book of words. The text is little more than a verbal matrix supporting the 220 coloured illustrations which form the really vital component of the work. Therein lie both its principal strength and its principal weakness: its strength, because a good series of colour photographs illustrating the histological features of the more important pathological lesions must obviously be of great value to the student of pathology; its weakness, because even this extensive series of pictures clearly cannot cover all noteworthy conditions and no verbal reference is made to those which are not illustrated. Thus even such familiar tumours as simple dermoids and papilliferous cysts of the ovary receive no mention, no renal lesions except nephritis and nephrosclerosis are recognised, brain abscess is forgotten and the very existence of testis and ovary, tube and uterus is virtually ignored. Such gaps make the book too patchy to be a satisfactory text-book of morbid histology, although it remains an admirable atlas.

Chief credit for the high quality of the illustrations belongs to Mr T. C. Dodds who made the original Finlay transparencies, but the sound work of the block-makers, Graphic Arts Ltd., is also deserving of recognition. There is admittedly a certain flatness in some of the low-power pictures but many of the high-power photographs rival good water-colours in clearness and vigour. The colouring of the stained

section is very faithfully reproduced and structural details are brought out in a manner that is seldom possible in the dull monochrome of ordinary photomicrographs.

In the text meticulous descriptions of histological changes are supported by brief accounts of naked eye appearances and frequent references to morbid processes. Most of the matter is orthodox enough but sometimes, as in the insistence upon phanerosis as the essential phenomenon in fatty degeneration, there is a tendency to recall the dogma of a past day. On the whole the histological descriptions are competent and clear but many passages suffer from a dullness and obscurity which certainly do not spring from an undue economy of words. Nevertheless the merits of the book greatly outweigh its faults and especially, as a pictorial record, it should prove of real value to the student of medicine, for whom it is primarily intended.

Architecture of the kidney in chronic Bright's disease

By JEAN OLIVER. New York and London: Paul B. Hoeber, Inc. 1939. Pp. vi and 257, 112 figs. including 5 in colour and 39 aquatone plates. \$10.

The introduction strikes a note which will be welcome to many pathologists, namely the fundamental importance of morphology as the basis of pathological function. This theme is developed throughout the book towards a completion of our knowledge of structural change in Bright's disease by the provision of three dimensional views of renal units in their continuity. The material employed is derived from cases which have been investigated jointly by Addison and Oliver, and symptomatology in relation to the changes appreciable by ordinary histological methods has already been considered by these authors in their monograph, *The renal lesion in Bright's disease* (New York, Hoeber, 1931). In the present volume from reconstructed models and from laborious micro dissections after acid maceration Professor Oliver has presented in stereograms surface photomicrographs and camera lucida drawings, many scores of completely separated nephrons and arterioles illustrating the varied distortions and adaptations which these have undergone in the course of prolonged disease. The need for brevity and not lack of appreciation forbids us saying more than that the technique which is evidenced in every process of preparation and representation is beyond all praise.

Descriptive matter begins with accounts of typical hypertrophic and atrophic units in a chronic glomerulonephritis. A large tubule goes with a large tuft and *vice versa* though this is not acknowledged as a functional correlation for even the hypertrophic tuft is considered to be almost out of action as a filter. It may be noted that the atrophic unit is not, advancedly atrophied: its tubule retains more than the average normal length and has a distinct lumen throughout while its tuft as represented remains moderately cellular. In the discussion of tubular changes evidence is given that alterations like fatty deposition or atrophy are the result of immediately local conditions—ischæmia or fibrous overgrowth—rather than of influences affecting the tubule as a whole.

The importance of obstruction by cast material in producing dilatations is also stressed. There is next a demonstration of the

development of aglomerular units, with all the appearance of functioning and even hypertrophic tubules, in late stages of the disease. This points to the substitution of a purely secretory process in the abnormal functioning, just as normally happens in fish with aglomerular kidneys. In the transformation of the arterial system the most significant change is the development of numerous new arterial twigs which may pass directly to intertubular capillaries, thus aiding the secretory function which is now allotted to the tubules.

An extensive and interesting section illustrates the "pattern of the kidneys" by drawings of numerous dissected nephrons from sixteen cases of chronic glomerulonephritis, four of arteriosclerotic nephritis and three of amyloid disease. In the glomerulonephritis a distinction is drawn between cases with only regressive changes and those showing partly regression and partly hypertrophy of nephrons; it is not found possible, however, to associate these subgroups with distinctive clinical pictures. In arteriosclerotic nephritis a notable feature is the survival of tufts with atrophy of the corresponding tubules.

The methods which have been employed in this investigation provide certainty of conclusion on many situations which remain debatable from ordinary histological data, while they afford new bases for attack on matters still unsettled. One main contention of fundamental importance is the untenability of the popular "glomerular atrophy—tubule collapse" theory in the evolution of contracted kidneys. Disputed on many grounds, this view is thought to receive a lethal blow by the demonstration of aglomerular tubules, and as a corollary the theory is propounded that in chronic nephritis pure tubular secretion tends to replace the normal mechanism.

While this difficult problem is impossible of full discussion, it appears to us that this contrary view is somewhat heavily reinforced by an assumption that many nephrons become "practically aglomerular," and that narrowed parts of first convoluted tubules are functionless though they may still possess lumina which could transport filtrate. It may be noted that out of 185 nephrons dissected from 16 cases of glomerulonephritis, 125 were sufficiently well preserved throughout to be isolated complete with tufts attached, while only 3 were pronounced aglomerular. On the other hand, markedly atrophic units were apparently impossible of dissection, thus remaining out of assessment. On this problem, however, as on other matters the wealth of freshly presented detail is woven into a comprehensible texture by the author's apt discussions and the challenge which these convey will only be taken up with safety by those who read and digest the arguments in their entirety.

The content of cells and proteins in the normal cerebro-spinal fluid

By AXEL V. NEEL. Copenhagen: Einar Munksgaard; London: Humphrey Milford (Oxford University Press). 1939. Pp. 141; 2 text figs. 7s. 6d.

Establishment of the normal is a necessity in all laboratory work and we are therefore grateful to Dr Neel for his painstaking study of the normal cell and protein content of the cerebro-spinal fluid. In his critical analysis of the work of others in this field he has explained how their figures may have been influenced towards greater latitude than he himself allows by the inclusion of cases which, although not neurological,

were under treatment for some condition which affected the cerebro spinal fluid. He emphasises the importance of psoriasis and hernia in this respect. More data of this kind are needed, and there is a half promise in the present book that the author may tackle this problem next. But until we know the possibilities and implications of abnormalities in the fluid in non nervous diseases the establishment of normal values has more theoretical than practical value.

Methoden der Virusforschung

By HENRIQUE DA ROCHA LIMA, JOSÉ REIS and KARL SILBERSCHMIDT. Lieferung 480 of Abderhalden's *Handbuch der biologischen Arbeitsmethoden*, Abt. VII, Teil 2. Berlin and Vienna: Urban und Schwarzenberg, 1939. Pp. viii and 384, 54 text figs. RM 24.60.

Those who have approached the study of the viruses from the realm of medical bacteriology should not be disappointed that more than half of this book is devoted to methods for the investigation of the virus diseases of plants. The remarkable advances made during the last few years clearly indicate that the worker in the field of animal viruses and his colleague who is studying the virus diseases of plants are converging upon the problem of the essential nature of these agents and neither can afford to be ignorant of the methods and results of the other. In this relatively small volume Professor Rocha Lima and his collaborators have collected much information that will be of value to all who are actively engaged in this work. The first few chapters dealing with cytological methods and the demonstration of elementary bodies are particularly good but it is surprising to find no mention of cell inclusions produced by agents other than viruses. The subjects of filtration and ultra filtration, centrifugation and the estimation of the size of viruses are fully covered. Descriptions are also given of the various physical methods by which the crystalline structure of tobacco mosaic and other plant viruses has recently been demonstrated. The cultivation of viruses *in vitro* and on the chorio allantoic membrane of the developing chick is treated in a section of 20 pages. A more detailed consideration of these important methods would have been justified. A bibliography is appended to each section. The book is well printed and contains excellent subject and author indexes.

Plant viruses and virus diseases

By F. C. BAILEY. Leiden, Chronica Botanica Company. London, William Dawson and Sons. 1939. Pp. x and 272, frontispiece and 36 text figs. Guilders 7 (17s. 6d.).

In recent years students of plant viruses have been joined by workers in subjects which at first sight seem to have little connection with plant pathology. Biochemists, physical chemists, serologists, statisticians and X-ray specialists have all taken a hand in the game and the ordinary biologist finds himself somewhat bewildered by these specialists each talking a jargon of his own. One welcomes therefore the present volume which gives a lucid explanation of much of this unfamiliar technique.

The introductory chapter is a clear and readable survey dealing with, among other points, the chief characteristics of viruses and the various views that have been held as to their nature. The question of the

classification and nomenclature of plant viruses is dealt with at some length; and while the reviewer does not agree with all the author's opinions on this question, a review is hardly the place to discuss what is admittedly a controversial subject. Chapter II gives a general description of the different kinds of symptoms caused in plants by viruses and includes an account of how symptoms can be used to estimate the virus content of a sample by what is known as the local lesion technique first developed by F. O. Holmes. Chapter III continues the description of symptomatology and contains a good account of the intracellular inclusions and a careful discussion of their ætiology. An illustration of intranuclear inclusions—a rare thing in plant virus diseases—is also given. Chapter IV deals with the transmission of plant viruses and also includes, for reasons not obvious at first sight, certain physical properties of viruses, reactions with enzymes and chemicals, and filterability. Chapter V is an adequate discussion of the relationship between plant viruses and their insect vectors. The use of the term "incubation period" to describe the delay in development of infective power within an insect, may be convenient but it is to be deprecated on the score that it suggests multiplication and the evidence in favour of the multiplication of plant viruses in the insect vectors is very slender compared to that in the case of animal viruses and their insect vectors.

In the next chapter the question of virus strains and their origin is discussed. The classical case of induced variation in a plant virus, thought comparable at one time to the production of vaccinia virus from smallpox virus, is the apparent attenuation of beet curly top virus by passage in *Chenopodium murale* and its apparent reactivation by passage in *Stellaria media*. Now, however, as foreshadowed by the author, a doubt has arisen as to whether the phenomenon may not be explained on the basis of selection of strains rather than an alteration in the nature of the virus. It is true enough however that strains of viruses can be produced artificially and do arise naturally and these questions are also considered. The question of acquired immunity is dealt with in this chapter; in plants, such immunity depends on previous infection either with the virus involved or a closely related one. In other words the plant need no longer be diseased but it must contain the virus or a strain of that virus in its tissues in order to be immune from further infection. Antibodies have never been demonstrated in plants, although some recent work on the acquired immunity of tomato plants to the curly top virus hints at such a possibility.

Plant viruses are exceedingly active antigens and chapter VII gives a clear and detailed account of their serological reactions. This is a subject in which the author himself has had considerable experience. The precipitin reaction is the one mainly used in such work but complement fixation, neutralisation of infectivity and anaphylactic shock have also been employed. Because of their specificity these reactions can be used in the rapid identification of plant viruses, as a quantitative test and even to gain some hint as to the shape of the virus particles by the nature of the precipitate.

Chapter VIII deals with the purification of viruses which commenced in earnest with the pioneer work of Stanley in the U.S.A. on tobacco mosaic virus. Three types of crystalline virus proteins have now been described, the paracrystals or microtactoids of tobacco mosaic virus, the dodecahedral crystals of tomato bushy stunt virus and the thin lozenge-shaped plates of tobacco necrosis virus. The various methods of purification used to produce these crystalline proteins—"salting out"

by chemicals and sedimenting the virus by the ultracentrifuge—are described.

In chapter IX some of the properties of the purified virus preparations, such as activity, measured serologically or by dilution, chemical composition, precipitation and isoelectric point are discussed. Chapter X is an interesting account of a subject unfamiliar to most pathologists and deals with the optical properties of purified virus preparations. It is rather curious that so many plant viruses, apparently unlike the animal viruses, are asymmetrical or rod-shaped and when made to flow are birefringent and show the phenomenon of "anisotropy of flow." Only three plant viruses—those of tomato bushy stunt, tobacco necrosis and tobacco ringspot—have been shown so far to have spherical or nearly spherical particles and, perhaps for this reason, it has already been demonstrated that two of them give rise to three-dimensional crystals in their purified form.

Virus workers have long puzzled over the size of virus particles, a subject discussed in chapter XI, and it is only in recent years that investigations of this point by the aid of ultrafiltration, the ultracentrifuge and X-ray measurement have given reliable results. Much of the earlier misconception of size has arisen from the dissymmetry of the particles being measured and from their tendency to aggregate.

In his opening chapter the author remarks :—"at the present time it seems most reasonable and probable that these nucleoproteins are the viruses themselves" and in chapter XII is gathered together the evidence supporting this thesis. The evidence now is such that most if not quite all virus workers will agree with the view that the nucleoproteins isolated from virus-infected sap are the viruses themselves. At all event: the onus of proof to the contrary rests with those who hold that more than one entity is concerned.

Chapter XIII deals with the physiology of virus-diseased plants, including the latest theories of virus movement in the plant. Chapter XIV discusses the combined subjects of classification and control and the book ends in chapter XV with a discussion of the theories concerning the origin and multiplication of viruses.

This is a good book, it is written in an interesting and readable style and the subject is presented in a coherent and consecutive manner and is not a mere statement of unrelated facts. The best chapters are those dealing with the serology and purification of plant viruses. These two subjects are recent developments and are unfamiliar to many, so that their lucid presentation is all the more welcome. The format of the book is attractive and it is clearly printed with few misprints. The illustrations are excellent and carefully chosen and there are two adequate indexes.

K. M. SMITH.

Analysis of handwriting: an introduction into scientific graphology

By H. J. JACOBY. London: George Allen and Unwin Ltd. 1939. Pp. 285; 161 figs. on 27 plates. 10s. 6d.

This book is well written and interesting, and the plausible and persuasive style of the author, who gives evidence of wide reading and extensive knowledge of his subject, renders it eminently readable though far from convincing.

He questions "the belief that certain traits of character were revealed by certain definite characteristics of handwriting" (pp. 36 and 37) and holds that "the correct interpretation of a handwriting is not an objective procedure in keeping with the principles of exact methods; on the contrary, the subjective personality of the interpreter is a decisive factor. All depends on his faculty of forming a character-portrait of the writer so that he can arrive at the only possible interpretation suggested by the given constellation of features" (pp. 39 and 40). "Handwriting, furthermore, consists of three distinct layers: a middle, an upper and a lower zone. . . . The middle zone, which is based on the line, is the central and essential part of handwriting. . . . The meaning of these three zones in handwriting corresponds to the division of the human personality into mind, soul, and body, and of the universe into heaven, earth, and the nether regions" (pp. 88 and 89). "Tendencies to the left in handwriting signify our attitude towards our own ego, tendencies to the right, our attitude towards our fellow beings, and . . . the left hand side from which the writing movement spreads symbolises, in terms of time, our past, our memories, etc., and the right hand side accordingly our future, our expectations, etc." (p. 194).

This is the pseudo-psychological basis upon which the writer builds his interpretations of handwriting, and he produces on some 25 pages a sample analysis of a handwriting which in its detailed delineation of the character of his subject reminds one of similar documents provided by palmists, phrenologists, etc.

The earnestness of the writer, his extensive knowledge of psychological terminology and theory and the large number of well produced illustrations of sample handwritings fail to convince one that the volume is a useful contribution to scientific literature.

JOSEPH SHAW BOLTON.

PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

12th JANUARY 1940

The sixtieth meeting of the Society was held in the Department of
Pathology, Cambridge, on Friday 12th January 1940

Communications and demonstrations

Those marked * are abstracted below

- CUTHBERT DUKES The spread of rectal cancer
- C V HARRISON Multiple myelomatosis with atypical systematized amyloidosis
- *L C MARTIN Observations on the histology of toxic nodular goitre
- A T H ROBB SMITH Eosinophil pneumonia
- *W M EL SADR Observations on the histopathology in rats following lack of different constituents of the vitamin B₂ complex
- J G GRENFIELD and T MOORE Nervous and muscular lesions in prolonged vitamin E deficiency
- JAL DUBASH O CLEGG and J M VAUGHAN Changes occurring in stored blood
- R J LUDFORD Growth characteristics *in vitro* of filterable and non filterable fowl tumours
- F J KING and THOMAS H BELT Silica content of tissues with and without silicotic lesions
- J STUART PATTERSON The recovery of *Listerella* from premature foetuses of ruminants
- J GORDON and J W McLEOD A simple method of identifying *Clostridium novyi* (*B. oedematiens*) in mixed cultures
- J H BURTON, J W McLEOD and T S McLEOD Some observations on R L Mayer's theory of the bactericidal action of para aminobenzene sulphonamide
- J W McLEOD and ANNA VON MAYR HARTING Observations on the bactericidal action of sulphanilamide and allied substances on anaerobic bacteria
- H L SHEEHAN Subendocardial haemorrhages in shock
- A S McFARLANE Electrophoresis of vaccinia virus
- O KHAIRAT and A A MILES The effect of a CO₂ atmosphere on blood cultures
- F H STEWART Some specimens of coliform bacteria in variation
- J R M INNES and J W WHITTICK Thrombo angitis obliterans in a horse
- E J KING Artificial standards for the estimation of blood urea

ROBERT KNOX. Colonial appearances of different types of *C. diphtheriae* on serum media.

THE GALTON LABORATORY SERUM UNIT. Blood groups and some other genetic characters.

R. I. N. GREAVES and A. N. DRURY. Preparation of concentrated human serum by drying.

H. R. DEAN and R. A. WEBB. Subendocardial hæmorrhage in anaphylactic shock.

A. M. BARRETT. Simple time-saving devices for counting reticulocytes and measuring red cell diameters.

F. BLAKEMORE, S. D. ELLIOTT and J. HART-MERCER. Streptococcal endocarditis in young lambs.

A. Q. WELLS. Intranuclear inclusion bodies in the kidneys of bank voles.

Abstracts

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OBSERVATIONS ON THE HISTOLOGY OF TOXIC NODULAR GOITRE

L. C. MARTIN

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The histologist can assess the clinical severity of primary thyrotoxicosis or Graves' disease from the amount and degree of diffuse hyperplasia present in the gland. This agrees with the clinical assessment in 80-90 per cent. of cases. Such a close agreement cannot be attained in toxic nodular goitre or secondary thyrotoxicosis, and the presence of areas of hyperplastic epithelium between the nodules is not considered to be reliable evidence of toxicity. Such areas can be found equally in clinically toxic and non-toxic nodular goitres, and it is suggested that this is a compensatory hyperplasia making good the thyroid substance disorganised by nodule formation. The fact is emphasised that hyperplasia of the thyroid epithelium is not a specific change indicative of thyrotoxicosis.

Clinical differences between primary thyrotoxicosis and toxic nodular goitre would support Hertzler's (1936) hypothesis of a different pathological basis for each, namely that the toxicity of primary thyrotoxicosis is one of epithelial hyperplasia, while that of toxic nodular goitre is one of degeneration, with a direct adverse effect on the heart. This hypothesis needs further confirmation, but is welcome as a sign of dissatisfaction with the unified pathological basis of primary thyrotoxicosis and toxic nodular goitre, founded on the presence of hyperplastic epithelium in both. It is suggested that a study of the differences, both histological and clinical, between the two types of goitre would advance the pathology of thyrotoxicosis further than continued efforts to strain the similarities.

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612 392 014 B₂ 616—091 8OBSERVATIONS ON THE HISTOPATHOLOGY IN RATS FOLLOWING LACK OF DIFFERENT CONSTITUENTS OF THE VITAMIN B₂ COMPLEX

M M EL SADR

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The complex nature of the heat stable vitamin B₂ was first indicated by the results of Chick and Copping (1930). Since then a number of its constituents have been described, of which riboflavin B₆ and the various "filtrate factors" of Lepkovsky and Jukes (1936) and Edgar and Macrae (1937) are universally accepted as factors essential for the nutrition of the rat. Riboflavin and vitamin B₆ have been obtained in pure crystalline form, while the filtrate factor is in a high state of purity. In the present work the pathological effects of deprivation of riboflavin, vitamin B₆ and filtrate factor have been studied separately.

The basal diet used was composed of pure substances adequate in all respects except for the vitamin it was desired to study. To obviate any effect of partial starvation due to loss of appetite, paired feeding was adopted in all experiments for deprived and control animals, i.e. paired litter mates were used as experimental and control animals and given the same food intake.

Deficiency of vitamin B₂ complex The rats refused food and lost weight after 50 days, at which time they did not show any specific symptoms except slight conjunctivitis and blepharitis. There was an average difference of 24 g body weight between the deprived and control animals. The thymus was completely atrophied, the pituitary, thyroids, suprarenals and spleen were relatively decreased in weight while the kidneys and liver showed increase in weight and severe hyperemia.

Deficiency of riboflavin The rats remained constant in weight for an indefinite period. After 2 months the eyes became affected with the following sequence of changes—conjunctivitis, blepharitis, diffuse corneal opacity, corneal cysts and cataract. The diffuse corneal opacity is probably due to fluid infiltration between the epithelial and subepithelial layers. There was complete atrophy of the thymus, the pituitary was diminished in weight while the liver showed an increase. There was no effect on the blood. When riboflavin was given, the diffuse corneal opacity disappeared within a few hours and after one week all lesions were cured except for the cataract, if this had been present. The anterior pituitary lobe was relatively increased in size and showed greatly increased vascularisation.

Deficiency of filtrate factor The rats grew at a subnormal rate and showed no specific symptoms except for depigmentation of the hair, the animals turning quite grey. This was cured by administration of a purified preparation of the fraction. No specific effect was observed on any of the tissues with the exception of the blood, animals which had been deprived for 40 weeks developed a hyperchromic macrocytic anemia. The average red cell count, percentage of hemoglobin and colour index were as follows—

	RBC	Hb (per cent)	CI
Deprived rats . .	6,400,000	98	1.5
Paired fed controls . .	9,310,000	93	1.01

Deficiency of vitamin B₆. Rats deprived of vitamin B₆ and fed on a basal diet containing sucrose as carbohydrate grew at a subnormal rate and developed the characteristic dermatitis of the ears. The thymus of some of the rats appeared completely atrophied and there was a relative atrophy of the pituitary, thyroids and suprarenals; the liver and kidneys were markedly increased in weight. The liver had a nutmeg appearance and showed fatty degeneration. There was proliferation of the Kupffer cells, which seemed to be actively phagocytic. The glomeruli of the kidneys were in some cases large and prominent, irregular in shape and richly cellular, while in others the basement membrane of Bowman's capsule was thickened and the epithelium of the convoluted tubules and both loops of Henle was degenerated. After about 100 days on the diet, the animals developed a microcytic, hypochromic type of blood. The average red cell count, percentage of hæmoglobin and colour index were as follows :—

	R.B.C.	Hb (per cent.)	C.I.
Deprived rats . . .	11,700,000	98	0.84
Paired-fed controls . . .	9,400,000	95	1.01

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 T. F.

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SEX HORMONES AND THE FOA-KURLOFF CELL

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(PLATE XXIV)

Our knowledge of this strange cell—the Foà Kurloff cell—in the blood of guinea pigs dates from 1889 when Foà and his pupil Carbone in Turin and Kurloff in Ehrlich's laboratory in Frankfurt independently described it. It has been most widely known as the Kurloff cell, probably because of the reference made to Kurloff's finding—originally published in a Russian journal—in *Die Anatomie* of Ehrlich and Lazarus (1898). Through the kindness of the library staff of the Royal Society of Medicine, the reference to Kurloff's original paper has now been traced (Kurloff, 1889), though so far it has been impossible to secure the actual journal for perusal. According to Ehrlich and Lazarus, Kurloff, in the course of a study of the blood of guinea pigs after splenectomy, described these peculiar cells as "Zellen mit Vakuolen", the vacuoles containing a nucleus like body staining with nuclear dyes. His impression was that the vacuole contained some "Sekretstoff" elaborated by the cell. An estimate he gave of the numbers of these cells in the blood of guinea pigs—15-20 per cent. of the leucocytes—would seem to be barely relevant, for we now know that the variations in their numbers according to age and sex are considerable.

The contribution of Foà and Carbone in the same year was more substantial. These authors in the course of a study of the cell types in the guinea pig spleen gave an accurate description of the cell in question and in the plate accompanying their paper a few quite typical examples are illustrated. The mere statement is made that similar cells are to be found in the circulating blood as well as in the splenic pulp and that they are much less numerous in the bone marrow. The nature of the inclusion body in these vacuolated cells puzzled Foà and Carbone as it has puzzled all interested observers in the intervening fifty years. "Sind es vielleicht Parasiten?" they write, and we are told that cultivation failed. The legend to the cells in the coloured plate is at any rate "*parasitenartige Körper*". In their hands the "inclusion" failed to give reactions for fat, albumen, starch, glycogen or iron. Perhaps their most important observation in the light of quite recent developments was that these peculiar cells were

very numerous in the spleens of pregnant females—an observation also made by the writer (Ledingham, 1906), then unfamiliar with the work of Foà and Carbone.

Their much greater frequency in the blood of the female and particularly the pregnant female has since been generally recognised.

The presence of Foà-Kurloff cells in foetal and early post-natal life

The writer (1906) reported that he had been unable to find definite examples of these cells in the blood of young guinea-pigs until about the third week of extra-uterine life. Cesaris-Demel (1905), on the other hand, claimed that they might make their appearance in some animals as early as the third day of life but in others not before the 20th day. In view of the writer's experience at that time, and of that of many subsequent workers up to quite recent times, it is probable that the considerably smaller size of the inclusion and its more compact form in the Kurloff cells of young and immature animals, together with their extreme scarcity necessitating long search, may have interfered with their recognition as Kurloff cells. The writer was in fact inclined to regard his inability to find these cells in the new-born and in the early days of life as indicating a real absence and many other writers since then have doubtless been similarly influenced. On returning to this subject in 1938 the writer was impressed by the claims of Senez (1922) and of Wada (1928) that they had detected undoubted Kurloff cells in very small numbers, sometimes at birth, sometimes at the end of the first week and on occasion even in the foetal spleen. Babudieri (1938a) has made extensive enquiry into this question and has reported that 6.3 per cent. of 220 guinea-pigs presented F-K cells in their blood at birth, while at the 10th day of life the percentage of guinea-pigs showing these cells rose to 20-25. The writer has confirmed these claims and can now state that provided at least one thousand leucocytes are counted in the fixed and stained films of blood taken at birth or in the early days thereafter, there is a good chance of finding one or perhaps two quite characteristic F-K cells.

While these cells are to be found in the circulating blood in infancy only after hard search, still harder search may be required to demonstrate them in impression preparations of the foetal spleen. They have been observed here, however, and like those in early infancy the inclusions in the vacuoles are small and compact. It may be allowed here to anticipate the main subject-matter of this communication by interpolating an experiment designed to determine whether F-K cells in foetal organs (blood and spleen) could be raised in number by administering œstradiol to the mother. A pregnant guinea-pig was given, on 8.3.39, 75 γ œstradiol dipropionate subcutaneously. Its F-K content on this day was

70 per mille On 18 3 39 the F-K content was 99 per mille and on the following day the animal was killed The uterus contained two fetuses approaching full time It was impossible to determine whether the foetal heart blood contained F K cells, as films taken *post mortem* revealed hardly any leucocytes, but splenic and bone-marrow impressions were made from both mother and fetuses The spleen impression from the mother showed 135 F K cells per mille leucocytes while those from the fetuses revealed 24 and 14 per mille None was noted in the foetal marrows It must be concluded therefore that F-K cells are normal constituents of the blood from foetal life onwards

Some morphological features which have impressed observers

The variety in form and dimensions assumed by the inclusion in fixed and Giemsa stained preparations (fig 1) has given rise to many hypotheses, some very fantastic, as to its nature and origin The appearance of the included substance in blood vitally stained with brilliant cresyl blue or neutral red (Cesaris-Demel, 1905, Ledingham, 1906) is however very characteristic With the former stain the substance appears as granular dark blue bodies showing brownian movement within the vacuole, while with neutral red it takes the form of more lumpy granules In both cases the granular matter takes up the stain immediately, so that the F-K cells are very readily picked up in vitally stained preparations Several authors including the writer have noted the effect on such vacuoles of downward pressure exerted by the objective on the coverslip and quick release The vacuole can then be seen to give way at one point, with escape of the stained granular matter Immediately this happens the stain adsorbed by the granules is discharged but the unstained granules can still be followed for a short period before they disappear into the surrounding plasma There would now appear to be little doubt that, as Cesaris Demel originally suggested, the variable forms assumed by the inclusion in the air dried film fixed in alcohol and variously stained are only reflections of the state of dispersion of the solid stainable material in the vacuolar fluid When however a strict routine of fixing and staining is employed, differences at different times in the amount of stainable matter in the vacuole and in the manner of its aggregation in the stained state, from large coarse skeins or granules, or filaments completely filling the vacuole, down to apparent emptiness, would seem to be correlated with age, sex and physiological states such as pregnancy This is a physico chemical problem and until we know something of the chemical constitution of the "inclusion" it would be unwise to speculate as to the precise significance of these varied forms An additional feature of the cell which is most striking in the unstained or vitally stained preparation, though not

invariably detected, is the presence in the cytoplasm surrounding the vacuole of small globules which appear as simple vacuoles in the fixed and stained film. Bender (1923-24) and Liggeri (1931) both claimed to have noted these for the first time and to have shown that they represented lipid droplets staining with sudan, but they were clearly described and figured by Cesaris-Demel in 1905 and the writer (1906) figured them without drawing special attention to them. Their origin and function are quite unknown.

Brief sketch of propounded theories of origin

Before proceeding with the experimental evidence on which a relationship of the Kurloff cell with the sex hormone system is based, little more can be done than to mention theories of origin put forward by various authors in the course of the past fifty years and readers who wish fuller details are referred to Babudieri's recent monograph (Babudieri, 1938a).

(A) *Parasitic theory.* This has had a great vogue and the inclusion has been taken to represent protozoa of various kinds, chlamydozoa and spirochæte-like bodies. This last suggestion was made by Ross (1912) as the outcome of his study of the Kurloff cell on agar jellies. So recently as 1937 Mochkovski of the Moscow Institute of Tropical Medicine confidently ranges the Kurloff inclusion with the *Rickettsias* and actually coins for it the name *Ehrlichia kurlovi* in honour of Ehrlich and of Kurloff who made the discovery in Ehrlich's laboratory. *Ehrlichia* would be a subgenus of *Rickettsia* and in it he would place also those Kurloff-like bodies alleged by certain authors to have been found in other animals than the guinea-pig, but it must be emphasised that the majority of writers who have searched for similar bodies in other animals, including even other species of *Cavidæ*, have completely failed to find them. Babudieri (1938a) has recently searched the blood of a long series of animals without success and decided that the inclusion is peculiar to the guinea-pig, as the writer (Ledingham, 1906) did after examining various members of the *Cavidæ* family housed at the Zoological Gardens, London. Etzel (1931) however claims to have seen similar bodies in Brazilian *Cavidæ* and the possibility should still be borne in mind that other members of the *Cavidæ* may harbour these structures. A single negative examination may be quite inconclusive, particularly if age, sex and the pregnant state are not taken into consideration. An adult male guinea-pig may show fewer F-K cells than one per mille leucocytes though it may immediately respond to the injection of œstradiol by exhibiting large numbers of F-K cells in the leucocyte population (see later). It would seem desirable therefore to establish the presence or absence of such cells in the *Cavidæ* generally by far more intensive studies.

The parasitic theory has no experimental evidence to support it.

(B) *Phagocytic theory.* Many authors have claimed that the inclusion represents phagocytised matter such as red cells or nuclear remains (Woodcock, 1921; Lazzeroni, 1935-36). Woodcock claimed to have obtained evidence of the presence of iron in the inclusion but most authors who have employed microchemical methods have failed to detect it.

(C) *Nuclear theory.* Perhaps the chief exponent of this view is Leinati (1932) who, on the strength of the not uncommon finding in guinea-pig blood of large lymphocytes containing two nuclear portions of equal size connected by a thin band of chromatin, claimed that one of the portions tended to degenerate by a process of karyorrhexis or pyknosis and so to become transformed into typical Kurloff bodies.

FOA KURLOFF CELL



FIG. 1—Types of Foa Kurloff "inclusions" met with in Giemsa stained films from various blood specimens. Note large coarse skeins, bacillary and granular forms, small compact types, sometimes multiple, together with almost empty vacuoles containing little or no stainable matter. Note also in some cases small empty vacuoles in cytoplasm surrounding vacuole (Cesaris Demel or Bender "lipoid" bodies) (M Rhodes del.)

(D) *Secretory theory.* It is now generally agreed that the cell containing the inclusion is not of the monocyte order but truly a member of the lymphocyte series. Blockade experiments with dyes have shown that the Kurloff cell does not behave like the monocyte, the cell *par excellence* which segregates dyes. In the majority of cases the Kurloff cell resembles the larger type of lymphocyte but often enough it barely exceeds in size the small lymphocyte. There has been much discussion as to whether the inclusion might represent some aggregation of the well-known azurophile granules so common in lymphocytes, in spite of the slight but definite tinctorial difference. Confusion might arise only in the case of the smaller Kurloff inclusions common in very young animals but it has been shown that, in conditions of increase of F-K cells following injection of sex hormones or of diminution following castration (see later), there is no concomitant rise or fall of the azurophile granules. In the new-born also, while Kurloff cells are extremely rare, azurophile granules are common enough constituents of the cytoplasm of lymphocytes. The theory that the Kurloff inclusion is some sort of secretion of the cell which harbours it, as originally suggested by Kurloff and a number of subsequent authors, would seem to fit best the recently acquired knowledge which brings the Foa-Kurloff cell into relation with the sex hormone system. Perhaps the first suggestion that the Kurloff cell might be so connected was made by Kolmer (1912) at the close of a communication dealing with the relation of the adrenal to sex function in the guinea-pig. Being aware of the fact that in pregnancy these cells underwent considerable increase, he threw out the suggestion that they might actually represent some secondary sex organ. Experimental research on this point dates from 1928 when papers by Alexeioff and Joukoff and by Wada appeared. The former castrated 60 animals—50 males and 10 females—and found that by the end of 5 or 6 weeks the Kurloff cells had disappeared from the blood but reappeared after 5-7 daily injections of "spermine." They further stated that in male guinea-pigs which had been castrated for as long as six months spermine injections failed to induce the reappearance of F-K cells. Wada, in the course of what would appear to be a mere summary in German of his studies on many features of the Kurloff cell problem, made a similar statement with regard to the effect of castration, and on the strength of it suggested that the inclusion was a physiological element dependent on the hormonal working of the sex glands, although closely related also to growth and development.

Following these authors, Semenskaja (1930) and Severi (1931), though recording undoubted diminution in number of the F-K cells after castration, found themselves unable to agree that complete disappearance took place. Discrepancies of this kind may be expected and may well be due to differences in the completeness of the surgical interference or in the thoroughness of the search for residual samples (see later). Cilotti (1931) on the other hand recorded complete disappearance of the F-K cells after castration and noted some return in response to injection of commercial preparations of the male sex gland or fresh guinea-pig testis. The communications of the workers quoted, on the effect of castration and of administration of sex hormones, together with those of a succession of Italian authors who entered this field during the years 1931-37 (Fiorini, 1933; Valle, 1933; Natucci, 1937; Tosatti, 1938, etc.), either contain no numerical data or data of so meagre a character to substantiate their statements that the present writer decided to explore this new development for himself. The employment by many writers of commercial hormone preparations of doubtful potency to promote increase of F-K cells in the normal or their appearance in the castrated animal was a further inducement to enter this field. Babudieri (1938a) alone, whose work

came to the writer's notice when he had well embarked on the problem, has put on record valuable data both on the sex hormone relationship and on many other aspects of the Kurloff cell problem which have been of great assistance in the course of this investigation.

EXPERIMENTAL DATA *

Methods. All figures for F-K cell content recorded on the charts as ordinates are based on counts of not less than 1000 leucocytes registered in Giemsa-stained films of blood removed as minute drops from the ear veins by sharp needle prick and immediately spread in thinnest film and with constant pressure in such manner as to provide upper and lower edges and tail for study. A regular routine was followed, counts beginning half-way along the upper edge, then proceeding to the tail which contains the majority of the white cells and must never be neglected—for the F-K cells may at times be very irregularly distributed—and lastly including a portion of the lower edge. Though other observers—*e.g.* Babudieri—have recorded the variations in the percentage of F-K cells among the non-granular leucocytes counted, it was early decided to treat the F-K cell for counting purposes as an autonomous member of the leucocyte population and not as a member of the lymphocyte series, to which on morphological grounds it would appear to belong. Along the base line are recorded the ages of the animals (all litter mates) at which observations were made.

Preliminary orientating experiments with œstradiol

Figs. 2, 3 and 4 record the results of certain orientating experiments commenced with other objects in view than the testing of œstrogenic substances. These were employed only at a late stage.

Expt. 1 (fig. 2). Two females born 24.12.38 and observed from the age of 12 hours. When this experiment was commenced, the writer was still under the belief that F-K cells were really absent in the early days of life and that the possibility of a post-natal infection giving rise to the peculiar inclusions was still worthy of consideration; hence the inoculation subcutaneously of both animals with a pleuropneumonia-like organism (isolated by Dr Klieneberger from abscesses in the necks of guinea-pigs) whose pleomorphic elements, if phagocyted, might reasonably reflect the varied forms assumed by the F-K inclusion in the fixed and stained state. Local abscess formation resulted but the infection did not induce the appearance of F-K cells. Not until the 15th day of life were F-K cells found, namely 4 per mille and 1 per mille respectively. Between the 15th and 38th day, when both animals received intraperitoneal doses of a rabbit *v.* guinea-pig serum in connection with another experiment, the F-K cells had reached levels normally found in non-pregnant females. On the 52nd day of life it was decided to test the effect of œstradiol dipropionate

* A brief abstract of these experiments has already appeared in the Proceedings of the Society (Ledingham, 1939).

in sesame oil suspension, 60 γ being injected subcutaneously in one animal while the other was left as control. The effect of this injection on the F-K content is obvious from the chart.

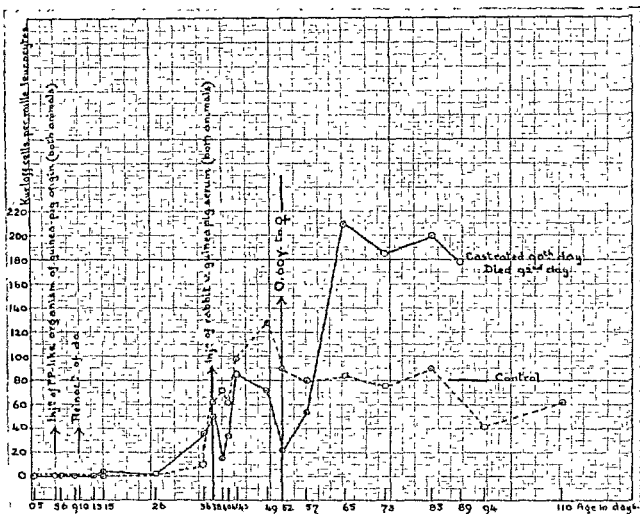


FIG. 2.—Litter (2 ♀) born 24.12.38 and observed from 12 hrs. Orientating experiment, œstradiol being administered on 52nd day to one of the animals.

Expt. 2 (fig. 3). Three females born 27.12.38 and observed from 24 hours. This litter (all females) was, as the chart (p. 208) shows, employed mainly for detection of the earliest appearance of F-K cells and it was not until the 49th day of life that the effect of injection of a sex hormone was tested, the one remaining animal being given 60 γ œstradiol. Observations thereafter were not systematically carried out but the enormous rise to 310 per mille noted on the 13th day and the subsequent fall to normal levels for the female showed conclusively the powerful specific action of œstradiol in inducing a large output of F-K cells.

Expt. 3 (fig. 4). Adult male, age unknown. This was the first animal on which the effect of œstradiol was tested (30.1.39). Like the animals of figs. 2 and 3 it had been used during Dec. 1938 for an experiment of a non-infective nature requiring differential blood counts. In such counts of about 400-500 cells no F-K cells had been seen. On 20th Dec. a count of 1200 cells was made and still no F-K cells were detected. On the following day, however, 2 were found

in a count of 1200 cells. Observations were resumed on 21st Jan., when counts of $<1\text{-}2$ per mille were recorded. It was therefore considered an excellent case on which to try the effect of œstradiol,

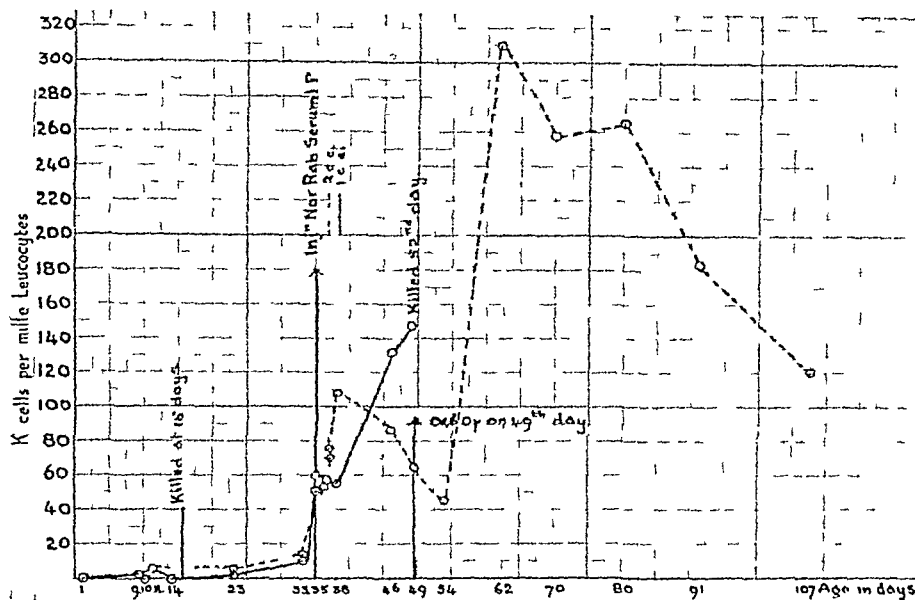


FIG. 3.—Litter (3 ♀) born 27.12.38 and observed from 24 hrs. Orientating experiment, œstradiol 60 γ being administered to one female on the 49th day of life.

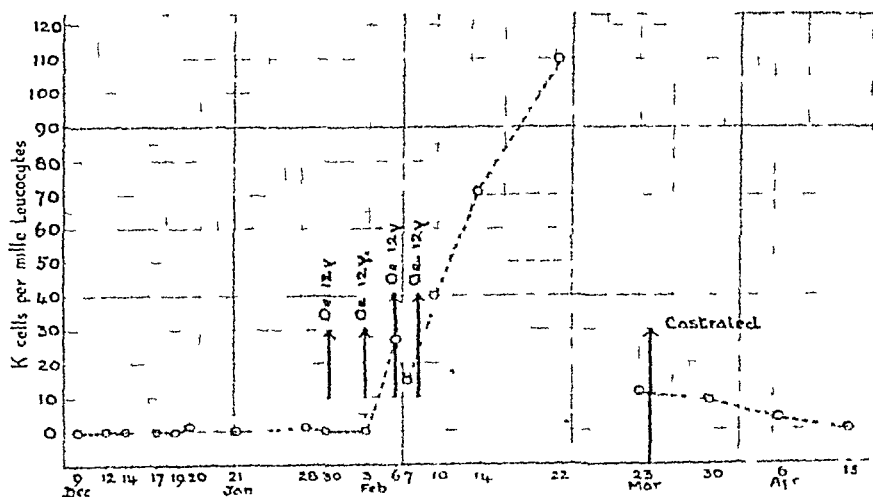


FIG. 4.—Adult male F-K cells estimated during three periods; 9th-20th Dec, 21st Jan.-22nd Feb, and 23rd Mar-13th Apr. Four injections of œstradiol between 30.1.39 and 6.2.39.

more particularly perhaps because the animal was a male and living in a cage by itself. Four injections of 12 γ œstradiol in sesame

oil suspension were given on alternate days between 30.1.39 and 8.2.39. The response was remarkable. On the 6th day the F-K cells had risen to 27 per mille and ultimately, on 22.2.39, i.e. 23 days after the first dose of the hormone, a count of 110 per mille was returned. On 23.3.39 the count had fallen to 12 per mille. The animal was castrated on 24.3.39 and subsequent observations are recorded partly in fig. 4 and partly in table II (animal B) on p. 216. The striking results obtained in these preliminary trials made it highly desirable to investigate thoroughly the action of oestradiol on males and females on the day of birth or on the early days of post-natal life.

For many reasons it was considered advisable to retain injected and control members of litters in the same cage with the mothers, though some risk of imbibition of hormone was undoubtedly incurred from suckling, licking, coprophagy or in other ways. The risk as tested by the F-K reaction proved to be slight.

Spaced injections of oestradiol

Expt. 4 (fig. 5). Litter of three (2♀ 1♂) observed from 24 hours. Both females at the age of 24 hours gave counts of

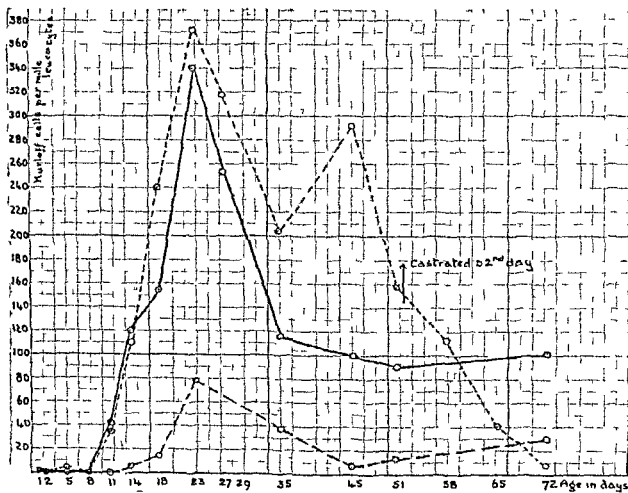


FIG. 5—Litter of three (1♂ 2♀) born 31.1.39 and observed from 24 hrs. Experiment to test the effect of spaced doses of oestradiol.

Upper curve, ♀, 9γ on 5th, 8th, 11th, 12γ on 14th and 60γ on 29th days.

Middle curve, ♀, 9γ on 5th, 8th, 11th and 12γ on 14th days.

Lower curve, ♂, no oestradiol

1 per mille while the male gave a similar count at 48 hours. The females received on the 5th, 8th and 11th days of life 9 γ α stradiol and on the 14th 12 γ , while one (see upper curve) received in addition 60 γ on the 29th day. The male was left as control. It will be noted that 6 days after the date of the first dose the F-K cells had risen to 41 and 37 per mille respectively and that the maximal peaks representing about one-third of the total leucocytes were reached 12 days later. The effect of the subsequent dose of 60 γ on the 29th day (upper curve) was not great and the curve proceeded on its downward slope to the 52nd day, when the animal was castrated (see later). The other female reached what appeared to be a steady plateau on the 35th day. The control male showed a small ascent and fall, most probably due to absorption of hormone from its sisters or mother or both.

Expt. 5 (fig. 6). Litter of three (2 σ 1 ϕ). Experiment planned similarly to that recorded in fig. 5. Here the female acted as

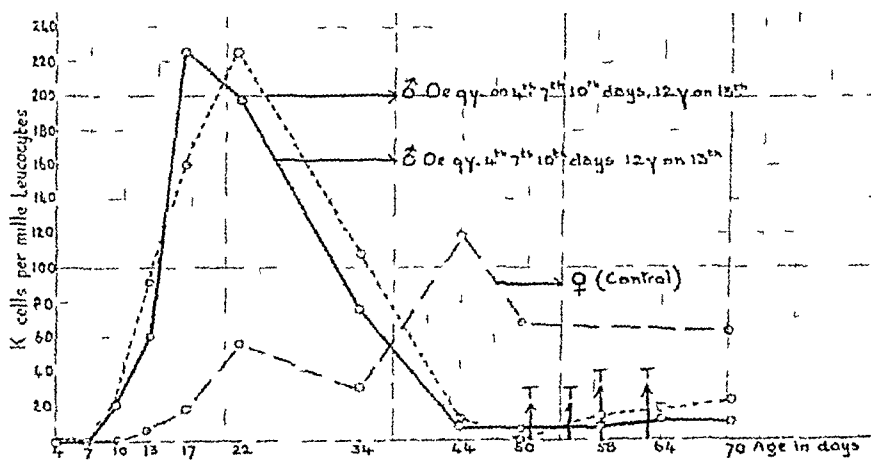


FIG. 6.—Litter (2 σ 1 ϕ) born 1 2 39 and observed from 4th day. Administration of α stradiol to the two males on 4th, 7th, 10th and 13th days, the female acting as control

control. Both males reacted strongly but the peaks reached were not so high as those attained by the females in fig. 5. Another important distinguishing feature was that the curves of the two males, unlike those of the females, fell sharply to minimal levels of 1 and 7 per mille on the 50th day. On the 51st, 55th, 58th and 62nd days testosterone (1500 γ) was given to these two males but the response was scarcely noticeable.

The effect of a single large dose of α stradiol given in the first week of life

Expt. 6 (fig. 7). Litter of four (3 σ 1 ϕ) observed from 6th day. This was an experiment designed to test the effect of a single dose

of 60 γ oestradiol in sesame oil and of the sesame oil menstruum itself. Two males which on the 6th day of life had given Γ K contents of 2 per mille and <1 per mille were given 60 γ oestradiol in 0.2 c.c. sesame oil, while the third male (Γ K content 1 per mille) and the female (Γ K content <1 per mille) received 0.2 c.c. sesame oil only. The chart shows that the male receiving sesame oil only did respond slightly, but almost certainly this rise was due to acquisition of hormone from extraneous sources. The female on the other hand showed little or no response and its Γ K curve proceeded on a rising course characteristic of the normal female to the 42nd day, when it was decided to try the effect of testosterone

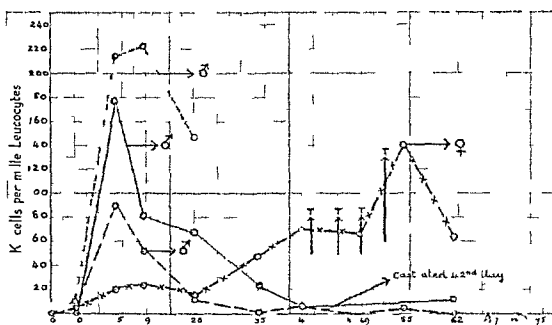


FIG. 7.—Litter of 4 (3 ♂ 1 ♀) born 10.2.39 and observed from 6th day. Experiment to test the effect of a single dose of oestradiol and of the menstruum—sesame oil—separately.

----- ♂ 60 γ O ———— ♂ 60 γ O ———— ♂ sesame oil
 -x-x-x-x- ♀ sesame oil Later 4 doses of testosterone (T)

in four doses of 0.2 c.c. (1500 γ), given on the 42nd, 46th, 49th and 52nd days. The effect of this was a rise from 71 to 140 per mille followed by a fall to a level normal for the female.

The effect of oestradiol injection on the day of birth in male and female

Expt 7 (fig. 8). Litter of two (1 ♂ 1 ♀). Each received on day of birth 9 γ oestradiol dipropionate. At birth the Γ K content of the male was <1 per mille and that of the female 2 per mille. On the 4th day the Γ K content of both male and female was still low, namely 3 and <1 per mille, but almost suddenly on the 6th day 47 per mille and 75 per mille were registered. Thereafter

the F-K content rose steadily, attaining in the male a maximum of 200 per mille on the 14th day and in the female one of 240 on the 22nd day. Again one observes the subsequent rapid fall of

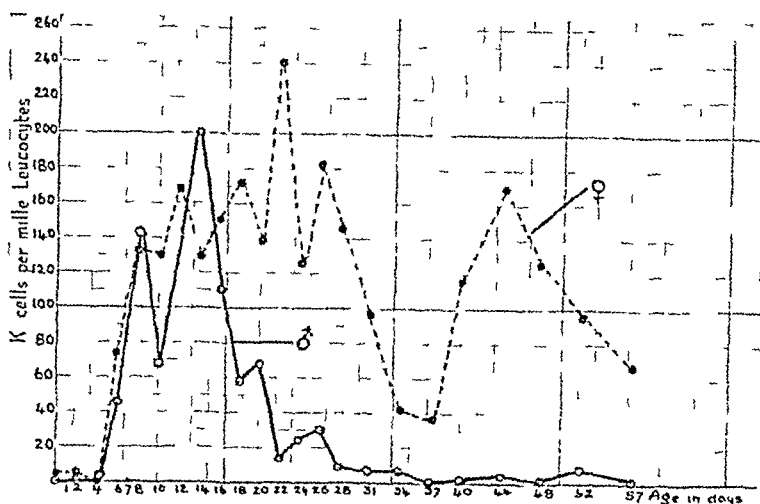


FIG. 8.—Litter (1 ♂ 1 ♀) born 21.4.39. Administration of oestradiol on day of birth.

the curve of the male to minimal levels while the female curve, after a secondary peak at 170 per mille, descended to a level expected in the female animal.

The effect of varying the dose of oestradiol given on the day of birth

Expt. 8 (fig. 9). Litter of three, all ♀. These were used in an attempt to compare the influence of 0.5, 3 and 9 γ oestradiol given on the day of birth. The female which received the smallest dose reached a peak on the 9th day while that receiving the largest dose attained a first maximum on the 12th day. Following the primary falls the curves crossed and recrossed and that of the female which received the smallest dose initially, rose to the very high level of 290 per mille on the 33rd day. The writer would interpret the curves as indicating that the specific primary action of the doses used was completed by the 15th or 18th day and that thereafter the course of the F-K cells with waves of increase and decrease was determined by local conditions of endocrine production and assimilation within the family. On the 46th day the animals which had received 3 and 9 γ were placed in separate cages while the one which had received 0.5 γ was left with the mother. Counts were subsequently made on the 52nd, 58th, 65th and 72nd days, with results as follows.

	0.5 γ	3 γ	9 γ
46th day (fig. 9)	169	206	174
52nd "	248	94	193
58th "	187	132	94
65th "	90	208	148
72nd "	309	165	186

The variations that can occur in the course of six or seven days are certainly remarkable.

Perhaps the initial stimulation to the sex organs by the injection of œstradiol on the day of birth gave an impetus to F-K cell

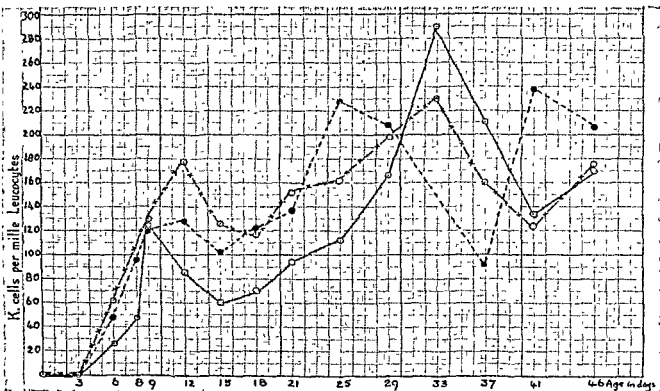


FIG. 9.—Litter (3 ♀) born 13.5.39.

— 0.5 γ }
 --- 3.0 γ } on day of birth.
 -x-x- 9.0 γ

production in these females which served to maintain the Kurloff cells at an unusually high average level for the normal virgin female, and such production appeared to take place in cycles. It has been claimed (Tosatti, 1938), though without illustrative protocols, that œstrous manifestations in the guinea-pig are accompanied by wide variations in the F-K cell content.

In any case the experiment was a failure in so far as the primary objective was concerned and in all probability the question would receive a more definite answer by the use, as test objects, of young males removed from the mother after the first few weeks of life. Extensive variations of the kind observed in this experiment have not been noted in males.

Injection of testosterone propionate and dipropionate followed by œstradiol

Expt. 9. The protocol of this experiment, which concerns a litter of six (4 ♀, 2 ♂), is reproduced in tabular form (table I) in

TABLE I

Effect of injection of testosterone propionate and dipropionate followed by œstradiol on the Fod-Kurloff cells

Litter of 6 (2♂ 4♀) observed from day of birth

Age in days	A (♀)	B (♂)	C (♂)	D (♀)	E (♀)	F (♀)
1	<1	<1	<1	<1	<1	<1
2.8	TP	TP	TP	TP	TP	TP
6	<1	2	1	2	2	1
10	8	1	2	1	3	7
14	8	6	7	<1	4	4
15	...	TP	TDP	TDP	TP	...
18	2	8	<1	1	2	2
22	<1	2	1	1	<1	<1
23	œ	œ	œ
27	4	2	5	6	3	<1
31	80	93	65	6	2	<1
35	263	150	180	15	5	5
40	219	161	200	68	5	<1
46	105	66	59	40	8	<1
52	195	38	39	127	52	16
59	111	10	18	115	98	27

TP = testosterone propionate 1500 γ.

TDP = testosterone dipropionate 1500 γ.

œ = œstradiol dipropionate 9 γ.

order to display more clearly than in a chart the very low F-K content of both males and females during the first month of life in spite of one or two injections of testosterone (1500 γ) given at the ages of 2.8 and 15 days. On the 23rd day three of the animals (A, B and C) were each given 9 γ œstradiol dipropionate and the response was immediate, female A reaching the highest figure and falling to a high plateau, while the two males (B and C) fell sharply to little over 10 per mille after attaining their maximal peaks. It would seem that testosterone in a single dose has little or no effect in raising the F-K cell content. It will be recalled that even in multiple doses at 3-day intervals (fig. 7) the effect of testosterone though definite was not remarkable and certainly not of the same order as that induced by œstradiol.

The effect of œstradiol on the F-K content of the progeny, when given to the mother before parturition

Expt. 10 (fig. 10). The mother of the litter of three (2♂ 1♀) received 30 γ œstradiol dipropionate 9 days, as it happened, before parturition.

At the age of 10 hours the young had F-K contents of 56, 57 and 74 per mille while the mother's figure was 36. The peaks were reached on the 7th day of post-natal life or 16 days after the administration of œstradiol to the mother, that of the female attaining, as usual, the highest maximum. The curves for the two males fell abruptly to 8 and <1 per mille on the 29th day while that of the female fell to a plateau representing 33 per mille on that day. It was then decided to test the effect of testosterone

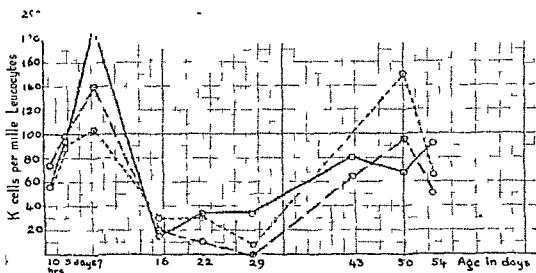


FIG 10—Litter (2 ♂ 1 ♀) born 23 2 39 Mother received 30 γ œstradiol 9 days before parturition

— ♀ Testosterone on 30th, 34th 37th and 41st days
 - - - ♂ do do do do
 ♂ No testosterone

dipropionate (1500 γ) on the female and on one of the males and four injections were given to each on the 30th, 34th, 37th and 41st days. The response of both animals was definite but not of a high order. The uninoculated male also showed a high peak on the 50th day, probably due to assimilation of hormone from a contaminated environment.

The effect of castration and of subsequent injection of œstradiol

Expt. 11. The animals chosen for this experiment, the results of which are given in table II, were three females and two males, the previous histories of four of which were known, namely (A) ♂ age 35 days on 17 3 (fig. 7), (B) ♂ adult (fig. 4), (C) ♀ adult virgin, age unknown, (D) ♀ age 45 days on 17.3 (fig. 5) and (E) ♀ multipara, mother of D.

On 17.3 and 23 3 counts were made on four of these animals—A, B, D and E. On 24 3 all five were castrated (both testicles or both ovaries) by Dr Korenchevsky, to whom the author's thanks are due. In the course of the following 7 weeks up to 13 5 the counts of A, B, C and D had fallen progressively to <1 per mille, while

that of the multipara still showed a significant F-K content. On 13.5 all were given 0.2 c.c. of sesame oil, which however had no effect on the F-K content, and a search of 1304 white cells in the case of the multipara revealed one F-K cell only. On 23.5 each received 9 γ oestradiol dipropionate in sesame oil.

TABLE II

Effect of castration and of subsequent injection of oestradiol on the Foà-Kurloff cells

Date	A (♂ age 35 days: fig. 7)	B (♂ adult: fig. 4)	C (♀ adult virgin)	D (♀ age 45 days: fig. 5)	E (♀ multipara: mother of D)
17.3	2	294	...
23.3	4	12	...	157	34
24.3	C	C	C	C	C
30.3	<1	9	37	114	34
6.4	9	41	17
13.4	<1	<1	4	9	20
28.4	<1	<1	<1	2	7
12.5	<1	<1	<1	<1	2
13.5	SO	SO	SO	SO	SO
19.5	<1	<1	<1	<1	<1
23.5	OE	OE	OE	OE	OE
27.5	<1	<1	<1	<1	<1
31.5	11	21	21	34	9
4.6	36	28	51	95	42
8.6	94	52	52	138	56
12.6	94	65	54	164	81
17.6	38	12	9	55	70
22.6	40	25	9	26	33
27.6	10	3	4	13	13
3.7	1	3	3	4	6
9.7	1	1	<1	<1	2
15.7	1	1	<1	1	<1
22.7	<1	1	<1	<1	1
1.8	<1	2	<1	<1	<1

Figures represent F-K cells per mille leucocytes.

C = castrated. SO = sesame oil 0.2 c.c. OE = oestradiol dipropionate 9 γ in 0.2 c.c. sesame oil.

All responded well, though the peaks reached were not so high as those attained by normal animals similarly inoculated in the early days of life. In little over a month the F-K content in both males and females had again fallen to the minimal levels obtaining before the administration of oestradiol. Unfortunately these animals were, by an oversight, destroyed a few weeks after the last counts had been made on 1st Aug. Otherwise it would have been possible to ascertain how long after castration an effective response to oestradiol could be obtained.

The effect on the F-K cells of the injection of urine from the pregnant human female

Fiorini (1933) and Babudieri (1938b) have reported that daily injection of young guinea-pigs with urine from pregnant women can

induce a very definite increase in the F-K cell content and that the method may be of value as a test for pregnancy. I have so far been able to complete only one experiment of this kind. The result however is sufficiently striking to merit inclusion here (fig. 11) pending further investigation of this aspect of the problem when circumstances permit.

Expt. 12 (fig. 11). Three samples of urine from females in advanced pregnancy were received from Dr Leonard Colebrook, to whom the writer's thanks for his kind offices are due. A pool was made and on 12.7.39 1 c.c. of the pool was injected subcutaneously into each member of a litter of two (1 ♂ 1 ♀) born on 30.6.39 and thus eleven days old. On the two following days the doses were repeated. Before the first injection the female had shown the somewhat high figure of 50 per mille while the male had

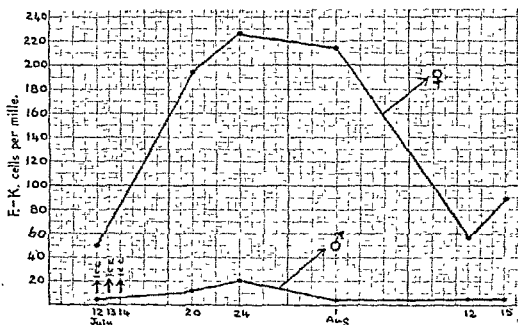


FIG. 11.—Litter (1 ♂ 1 ♀) born 30.6.39. Experiment to test the effect of injection of urine from the pregnant human female, both animals receiving 1 c.c. on three successive days—12th, 13th and 14th July.

only 3 per mille. Eight days after the first injection the F-K content of the female had risen to 194 and four days later had reached a peak of 227, after which there came the usual slow decline.

The male by contrast responded poorly, even allowing for the initial difference in F-K content. Unlike œstradiol, which invariably induced in the male a rise in the F-K content definitely but not markedly inferior to that induced in the female, the stimulating substance in human pregnancy urine appeared to influence the F-K content of the female only and, in this experiment at least, showed itself little inferior to œstradiol.

DISCUSSION

This renewed study of the Foà-Kurloff cell, undertaken after an interval of 32 years with the object of testing carefully certain

recent claims pointing to their dependence on the integrity of the sex organs and their quantitative regulation through impulses proceeding from the sex hormone system, has served to confirm quite definitely these claims. Castration reduces these cells to negligible numbers in the circulating blood and there is good evidence that artificial stimulation of the castrated animal, particularly with oestrogenic substances, can cause these cells to reappear in large numbers for a time. How long after castration an animal is able to respond in this way to stimulation by oestrogens is not yet certain. Further, it is now perfectly clear that both in the foetus and in the new-born animal there is no real absence of F-K cells, though it may require long search to detect them. At this stage both male and female animals react most powerfully to the injection of oestrogenic substances, particularly oestradiol, but, so far as this work has gone, much less powerfully to testosterone. So powerful is the response to oestradiol that we may shortly be in a position to employ chemical methods designed to ascertain the precise nature of the inclusion that gives to the F-K cell its characteristic appearance. It has been shown that injection of oestradiol can raise the F-K cell content to numbers representing about 40 per cent. of the total leucocyte population. Further knowledge of the nature and function of the Foà-Kurloff cell must, indeed, await such chemical enquiry. Should it be possible to isolate the substance of the inclusion chemically and to show that it possesses oestrogenic properties or the capacity to induce in the blood of young guinea-pigs or of castrated animals the appearance of Foà-Kurloff cells, we should be in a better position to appreciate the significance of a cell system acting as an apparently unique accessory to the sex endocrine apparatus of the guinea-pig. On the other hand it might emerge that the substance of the inclusion represents some breakdown product on its way to dissolution in the spleen, where undoubtedly these F-K cells largely congregate.

SUMMARY

A study has been made of the response of the Foà-Kurloff cells to injections of sex hormones, particularly oestradiol, and to castration. Charts and tables are presented which amply confirm the claim that, whatever their precise function may be, their quantitative variations in the blood of guinea-pigs both male and female depend on the integrity of the sex organs and hormonal influences proceeding therefrom.

I am greatly indebted to Dr Korenchevsky for supplies of sex hormones employed in this experimental study and for advice as to their manner of administration.

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FIBROSIS OF THE LIVER IN HEART FAILURE

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(PLATES XXV AND XXVI)

THERE is no general agreement as to whether chronic passive congestion may lead to fibrosis of the liver. Salaman (1907) showed that repeated attacks of heart failure render the liver increasingly resistant to distension by fluids when injected under pressure. This change was accompanied by a histological picture of fibrosis. Cornil and Ranvier (1884) used the term "cirrhose cardiaque" to describe a condition of fibrosis in the centres of the lobules and around the branches of the hepatic vein which they thought to be the result of heart failure. On the experimental side Zimmerman and Hillsman (1930) demonstrated by serial biopsies an increase in connective tissue in the liver of dogs following partial occlusion of the inferior vena cava. On the other hand Mallory (1911) denied that chronic passive congestion could result in a true increase in connective tissue in the liver. In his view the appearance of fibrosis was produced by shrinkage and thickening of the connective tissue fibrils in the centres of the lobules. Lambert and Allison (1916) were of a like opinion. Bolland and Willius (1938) also described the condensation of reticulum in the centres of the lobules. They consider true cirrhosis to be rare in association with heart failure and to be the result of the inclusion of portal fibrous tissue in extensive areas of damage.

Hersheimer (1908) described an increase in reticulum fibrils in the centre of the hepatic lobules in severe and long standing cases of heart failure but denied that this amounted to cirrhosis. He maintained that true cirrhosis when it is found in conjunction with heart failure is not the result of blood stasis alone but is due to additional factors.

In most cases of chronic heart disease the effect on the liver is not constant but intermittent. The regenerative capacity of the liver is high and it has been proved that the effect of intermittent damage is not necessarily permanent. Cameron and Karunaratne (1936) have shown that while repeated doses of carbon tetrachloride will produce cirrhosis in the liver of the rat, this will not occur if the time spacing is sufficiently lengthened to allow recovery between each dose. To determine whether passive congestion may itself result in hepatic fibrosis it is therefore necessary to take into account such factors as the severity and duration of each attack of heart failure, the number of attacks and the periods elapsing between. In practice such data are very difficult to get.

It seemed therefore of interest to examine the histology of the liver in cases where the state of congestion had necessarily been persistent for some time before death. Such cases are rare and it

is due to the kindness of Professor H. M. Turnbull that we have been able to use for this research material collected over a period of many years at the Bernhard Baron Institute of Pathology at the London Hospital.

The material from which this survey was drawn was obtained from 8 cases of constrictive pericarditis and 3 cases of long-standing right heart failure. The cases of right heart failure without pericarditis were of a diverse nature but each presented a picture of enormous hypertrophy of the right ventricle and auricle. The first was a case of severe mitral stenosis associated with a patent foramen ovale so large that both auricles formed in reality one cavity. The second was a case of mitral stenosis with tricuspid incompetence in which before death pulsation of the liver had been noted. The third was a case of immense hypertrophy of the right heart associated with atrophic emphysema of the lungs. This patient had been under observation and proved to have had a constantly raised venous pressure for at least two years. All these cases had suffered from ascites during life.

There is one feature common to every member of these two groups. The pressure in the upper end of the inferior vena cava, as elsewhere in the venous system, must in each case have been raised. The raised pressure persisted without intermission over a very long period of time. In most cases the venous pressure had been actually measured during the life of the patient; in the remainder a long-continued rise could be inferred from the condition of the heart at autopsy.

Histology

In contrast with the usual diffuse reticular type of fibrosis, the changes here described are not uniformly distributed throughout the liver. The most severe degrees of change occur around the larger branches of the hepatic veins and beneath the capsule. In general they are more intense towards the peripheral parts of the liver. Another difference is the rarity of nodules of regeneration. Areas of liver may appear as such but closer examination will show the constituent lobules are not in fact increased in size nor is their architecture distorted. A general reduction in the size of the lobules as the result of central atrophy is more usual.

In the affected areas there is an increase in connective tissue which is characteristic in its relation to the lobular pattern. The fibrosis occurs at both peripheral and central parts of the lobules (fig. 1) and there are numerous strands which connect the central and peripheral networks. Inasmuch as the new-formed fibrous tissue encroaches on the lobules it is invariably associated with a severe degree of parenchymatous atrophy. There is also an exaggeration of the normal fibrous markings, both in the portal systems and around and in the walls of the central veins.

In the normal liver there is an extension of fine connective tissue fibrils from the adventitia of the central veins outwards into

CONGESTIVE FIBROSIS OF LIVER

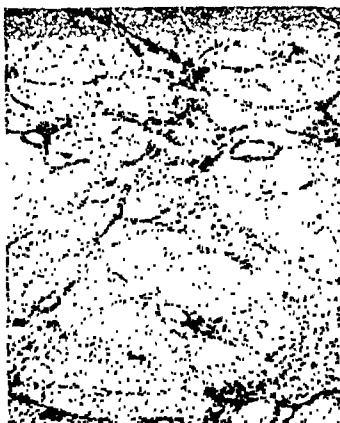


FIG. 1.—Low power view showing general distribution of fibrosis, which involves both the central and peripheral parts of the lobules. Mallory's triple stain.

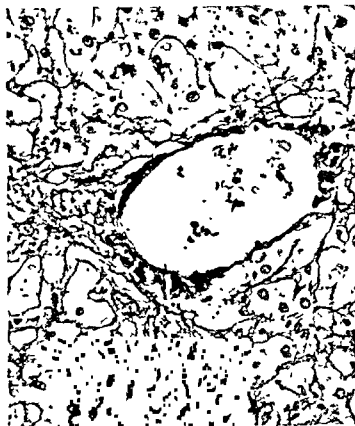


FIG. 2.—Proliferation of network of fine reticulum fibrils around central vein of lobule. Wilder's silver impregnation method.



FIG. 3.—Fibrous thickening of a branch of the hepatic vein. Note the network of fine fibrils in the oedematous subintimal tissue. Mallory's triple stain.



FIG. 4.—Great increase in elastic tissue in a branch of the hepatic vein. The elastic fibrils also extend into the surrounding lobules. Sheridan's elastic stain and neutral red.

the lobules. Such fibrils, called "Radiärfasern" by German authors, are usually increased in thickness. There is also a proliferation of finer fibrils arranged in delicate networks (fig. 2). The minute size, number and staining properties of these fibrils suggest that they are of new formation. The degree of separation of the fibrils is also evidence against the view that the fibrillary increase in the centres of the lobules is due to compression and collapse of the parenchyma and so only apparent. The central fibrillary increase is not accompanied by a proportional proliferation of connective tissue cells. Inflammatory infiltration is also rare.

There are similar extensions of fibrous tissue into the lobule from the portal tracts. In either case the fibrosis is accompanied by atrophy of the liver cells and it is a striking feature that the distribution of both atrophy and fibrosis is usually eccentric. Segments of lobules become involved and lead to the formation of connective tissue bands which unite the portal connective tissue with that around the hepatic veins.

There is a general increase of collagenous and elastic fibrils within the portal tracts, leading to exaggeration of the tracts without fibrous encroachment on the lobules. It is usually accompanied by an increase in fibroblastic and chronic inflammatory cells. Proliferation of "bile pseudo-canaliculi" occurs but is not as marked as in ordinary portal fibrosis.

The hepatic veins and their branches are always thickened in some degree (figs. 3 and 4), due partly to oedema and partly to an increase in elastic and collagen fibrils. In the media and adventitia these are stout and densely stained. Beneath the intima they are fine and are best demonstrated by silver impregnation methods. The subintimal increase leads to polypoid protrusions into the lumen which frequently lead to complete occlusion. This change is not accompanied by cellular proliferation or infiltration and the term endophlebitis as applied to it in a recent paper by Coronini (1939) would seem to be incorrect. It is more probable that the subintimal thickening is primarily due to oedema.

It has been mentioned that the process of atrophy and fibrosis is most marked in the peripheral parts of the liver. The capsule may therefore become thickened by the complete disappearance of the liver cells in the subjacent parenchyma and their replacement by bands of connective tissue (fig. 5). On the other hand there is frequently a deposition of collagen fibrils on the outer aspect of the capsule. This is shown very well in sections stained for elastic fibrils, since the elastic tissue in the capsule itself stands out clearly in contrast to the collagen deposited outside it (fig. 6). Oedematous polypoid tags of almost acellular collagen frequently project from the capsule (fig. 7). These are identical in appearance with those described by Bolton and Barnard (1928) in their work

on experimental occlusion of the inferior vena cava. It must be emphasised that the capsule in these cases was thickened by masses of relatively acellular collagen. Inflammatory infiltration and cellular granulation tissue of the kind which occurs in true polyserositis were not seen.

There is always marked evidence of lymphatic dilatation and œdema throughout the entire liver. The lymphatics in the capsule and in the portal spaces (fig. 8) are dilated and there is a wide separation of the connective tissue fibrils indicating œdema. Œdematous changes in the central and hepatic veins have been described above. Widening of the space which lies between the trabeculae and the endothelium of the sinusoids, usually considered to be diagnostic of hepatic œdema, is seldom seen. More usually there is a striking and often quite lacunar dilatation of the sinusoids themselves. They are often devoid of blood cells or are entirely filled with a homogeneous material which stains faintly with aniline blue. Similar material may also be found in the dilated lymphatics.

The histology of the liver in this condition may be summarised therefore as a fibrosis affecting both central and peripheral parts of the lobules, having its maximal incidence beneath the capsule and around the branches of the hepatic vein and accompanied by parenchymatous atrophy. Cellular increase either in the form of parenchymatous regeneration or interstitial inflammatory infiltration is remarkably slight. There is always evidence of œdema.

Discussion

In each of the 11 cases studied, a persistently raised venous pressure resulted in fibrosis of the liver of a highly characteristic type. The constancy of this finding indicates the direct connection between the rise of venous pressure and the increased production of fibrous tissue. Moreover, were the fibrosis the result of an added toxic factor it would be expected that the resulting histological picture would resemble that of an ordinary toxic cirrhosis. This is not the case nor does the type of fibrosis here encountered resemble that of portal fibrosis modified, as it often is, by the effects of heart failure. Obliterative changes in branches of the hepatic vein were encountered but we dispute the contention of Coronini that this change is necessarily inflammatory or indicative of a superadded infective or toxic process. The thickening of the veins is due to fibrillary increase and œdema without cellular proliferation.

How the mechanical fact of passive congestion may result in fibrosis is speculative, for the mode of formation of connective tissue fibrils is itself still disputed. There is a growing belief that fibrils are not the direct product of connective tissue cells but are formed extracellularly out of an amorphous substrate. Duguid

CONGESTIVE FIBROSIS OF LIVER

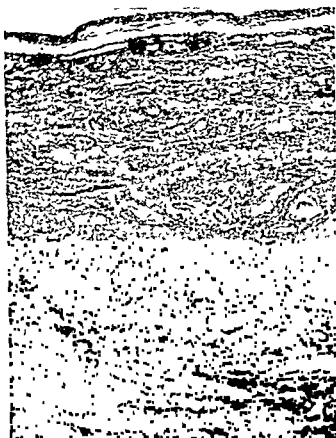


FIG. 5.—Thickening of the capsule due to atrophy and fibrous replacement of the subjacent parenchyma. Sheridan's elastic stain and van Gieson.

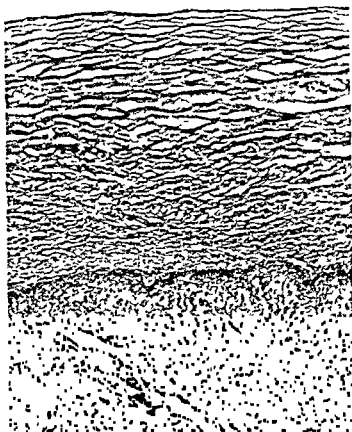


FIG. 6.—Thickening of the capsule of the liver due to hyaline collagen fibrils deposited on the outside. The position of the pre-existent capsule is shown by the darker elastic fibrils. Sheridan's elastic stain and van Gieson.



FIG. 7.—Fibrillary tags on the capsule of the liver. Note the lacunar dilatation of the sinusoids in the subjacent parenchyma. Wilder's silver impregnation method.



FIG. 8.—Dilatation of lymphatics in a thickened portal space. Iron hæmatoxylin and van Gieson.

(1938) has shown that even in the frankly inflammatory condition of experimentally produced nephritis there is a disproportion between the increase in connective tissue fibrils and the proliferation of interstitial cells

From a study of the appearances of connective tissue fibrils in various pathological conditions, one of us (Day, 1936), concluded that blood vessels exude a plastic substrate out of which fibrils are subsequently formed. It was suggested that fibrosis unaccompanied by cellular proliferation is likely to occur when, as in oedema, the tissue spaces become chronically distended by such a substrate. A condition of chronic distension of the tissue spaces is undoubtedly present in the condition described above and it is suggested that this is the main reason for the increase in fibrous tissue. It is of interest that Rossle (1933) has described a form of fibrosis of the liver occurring in Graves' disease accompanied by pericapillary oedema but by no proliferation of connective tissue cells ("serous hepatitis"). He assumed that the oedema was due to a thyrotoxic effect upon the capillary endothelium.

Another factor leading to the fibrous induration of organs is parenchymatous atrophy. This is well illustrated by the ischaemic atrophy of the kidney occurring in chronic cardiovascular degeneration. Here the decrease in size of the convoluted tubules is accompanied by a dense acellular thickening of the peritubular fibrous tissue. It is also common experience that meat becomes increasingly tough with the age of the animal eaten. It has been shown that within the lobule and particularly around the central vein the fibrillary increase coincides with parenchymatous atrophy both in degree and in distribution. It was observed that fibrillary increase, both in the thickness of pre-existent fibres and in the formation of new ones, occurred in the potential space which exists between the liver cells and the endothelium of the sinusoids. It must be assumed that the process was at some stage aided by the distension of this space by oedema fluid but a marked separation of the endothelium from the trabeculae was not usually noted. This may be due to the fact that the condition was only seen in its terminal stage. In the capsule and portal spaces and in the walls of the central and hepatic veins there is an increase in connective tissue fibrils which does not involve replacement of pre-existent structures. This we assume to be the result of the widening of the tissue spaces by oedema, with subsequent fibrosis. It is particularly well shown in the branches of the hepatic veins, where increase of silver-stained fibrils in the oedematous subintimal tissue leads to the formation of projections which encroach upon and sometimes occlude the lumen. A similar increase in fibrils occurs in the oedematous tags which project beneath the endothelium of the capsule.

The theory of extracellular fibrillogenesis does not imply that cells take no part in the process. On the contrary, tissue culture workers (Doljanski and Roulet, 1933) have put forward suggestive evidence that the mesenchymal cells are necessary in that they elaborate a diffusible stimulus which evokes the formation of fibrils in the surrounding substrate. The chronic excess of such a substrate in the tissue spaces may be expected to result in the production of an increase in fibrous tissue without necessarily involving a proliferation of cells. In this way a purely mechanical event such as a persistent rise in venous pressure may lead to fibrotic changes. This is particularly so in the liver. Starling has shown that when the inferior vena cava is obstructed above the diaphragm the flow of lymph in the thoracic duct is enormously increased and that this increase comes entirely from the liver. In the somewhat similar circumstance of a persistent increase in venous pressure the tissue spaces will become distended with fluid which, if our theory is correct, will provide the necessary substrate for the increased production of connective tissue fibrils.

Summary

Eleven cases were investigated in which a persistent rise in venous pressure was associated with fibrosis of the liver of a highly characteristic type. This was not the result of an inflammatory reaction to toxic or infective agents but must be considered to be the direct result of the altered hæmodynamic conditions. The role of œdema in the production of fibrosis is discussed.

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THE OCCURRENCE OF VIRUS III IN RABBITS IN THE LESIONS OF INFECTIOUS FIBROMA AND OF A TRANSPLANTABLE SARCOMA

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(PLATE XXVII)

VIRUS III AND INFECTIOUS FIBROMA

THE infectious fibroma of rabbits (Shope, 1932) has been studied in this laboratory since 1933 ; during this time the virus (OA strain—Andrewes, 1936) has undergone 45 serial passages through rabbit testes. Almost all these testes have been examined histologically, but intranuclear inclusions were never observed until September 1938, when they were found in small numbers in the otherwise typical fibromatous testis of a rabbit (no. 14) killed 16 days after inoculation with fibroma virus. This rabbit had been used 26 days previously for intramuscular transplantation of rabbit sarcoma RS 1 (Andrewes and Ahlström, 1938) but the graft was unsuccessful.

After storage in glycerol at -2° C. for 3 weeks a suspension of rabbit 14's testis was inoculated intratesticularly into rabbit 35 ; this rabbit was killed after 6 days and small numbers of intranuclear inclusions were found histologically in this testis also ; after one further passage to rabbit 141 a testis was obtained in which almost every cell of the fibromatous tissue contained an intranuclear inclusion. These inclusions consisted of an eosinophilic mass separated by a halo from the marginated basophilic chromatin. Their appearance recalled very strongly the inclusion body of rabbit virus III (Rivers and Tillett, 1923, 1924) and in fact they proved to be caused by that virus.

Virus III is a virus indigenous amongst certain stocks of rabbits. Rivers and Tillett found that 20 per cent. of rabbits in New York in 1924 were naturally immune to it. In 1928 I failed to find the virus at all in London rabbits (Andrewes, 1928) though McCartney (1925) met it in Edinburgh. Recently it has been hard to find in America.

The symptoms of the natural disease in rabbits are unknown ; possibly it is normally a symptomless infection. It is brought to

light by serial intratesticular passage in rabbits, preferably at 4-day intervals: after a few such passages through apparently normal American rabbits the agent "appears," giving rise to fever and acute orchitis. On intradermal inoculation it produces erythematous lesions which are scarcely raised above the surface. Normally it ceases to be recoverable from infected testes after about the sixth day, but in the Brown-Pearce carcinoma Rivers and Pearce (1925) found that it could persist indefinitely, being carried along in tumour transplants though these were made at long intervals.

Identification of the cause of the inclusion bodies in fibromata

It proved unexpectedly difficult to separate another infectious agent from the fibromata which contained inclusions. Pure fibroma virus free from the other agent was, however, readily obtained from a sample of fibroma material which had been kept in glycerol for 326 days; no inclusions appeared in the lesions produced by this strain. Dr T. M. Rivers sent me some glycerolated virus III. Sera from two rabbits which had recovered from the fibroma-with-inclusions neutralised this virus III when mixtures were inoculated intradermally, while sera from two rabbits immune to "pure" fibroma virus failed to do so. Rabbits immune to the supposed double infection were also immune to virus III. Further, a rabbit immune to virus III formed no inclusion bodies when inoculated intratesticularly with the supposed mixture; only fibroma lesions were produced. These cross immunity tests indicated that the unknown agent in the fibroma was virus III.

Serial passage of the double infection

The fibroma-with-inclusions was passed serially through ten rabbits: no attempt was made to propagate it further. Four- to ten-day intervals proved suitable for passage. The fibroma-like tissue in a rabbit testis infected with fibroma virus is derived from the interstitial tissue and capsule of the testis, the same tissue in fact as is attacked by virus III. In the third serial rabbit (no. 141 already mentioned) large areas of fibroma tissue revealed an intranuclear inclusion in every cell. In only a few microscopic fields was there fibroma without inclusions. Some of these inclusions stained purplish with hæmatoxylin and eosin and there was no halo between the purple mass and the marginated basophilic chromatin, but usually the halo was very definite and the central mass stained pink. The appearances differed from those typical of virus III infection in that the cells attacked were highly abnormal in shape, having many of the characters of an anaplastic new growth (fig. 1). Situated as they were in abnormal nuclei, the inclusions themselves were often of bizarre shape and varied size. Some were apparently

VIRUS III IN RABBIT TUMOURS

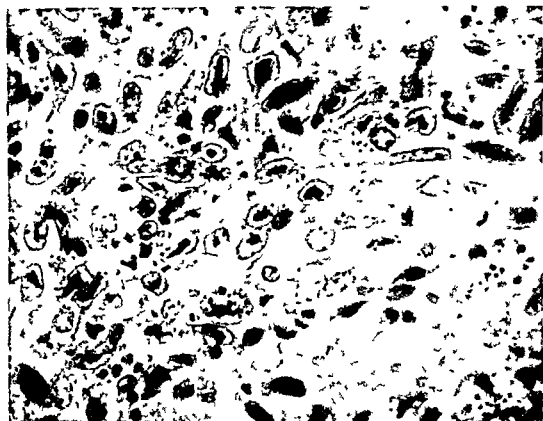


FIG. 1—Intranuclear inclusion bodies in fibroma tissue, they are of very varied shapes and sizes $\times 450$

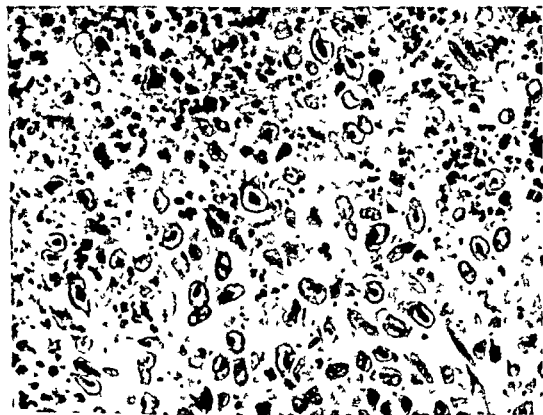


FIG. 2—Intranuclear inclusion bodies in sarcoma tissue at the margin of a necrotic area. The inclusions are more uniform in character than those in fig. 1 $\times 450$

lying free in the tissues, the nuclei containing them having probably disintegrated

Passages of virus III through normal rabbits must be made at not more than 4- or 5 day intervals if the virus is not to be lost. Yet transfers could be safely spaced as widely as 9 or 10 days in the case of the fibroma with inclusions. This fact recalls the long persistence of virus III in a rabbit carcinoma (Rivers and Pearce) and suggests that in rapidly dividing cells virus III may persist longer without causing the death of the cell, thus encompassing its own destruction. Intranuclear inclusions were found in fibroma lesions in two rabbits as late as the 16th and 17th day after inoculation, but when the fibromata had regressed and contracted (27th and 30th day in the two rabbits) no inclusions were found and virus was not recoverable.

The effect of virus III on the infectious fibroma

It might be expected that a necrotising virus such as virus III would have an adverse effect on the growth of fibroma lesions. Such proved to be the case. Passage from fibroma testes with abundant inclusions produced in the inoculated rabbit scarcely recognisable fibroma lesions—and not many virus III inclusions. In fact rapid passage led to the almost complete disappearance of both agents. It was only possible to keep the fibroma-with-inclusions going by making use of the fact that virus III is much less stable on keeping than fibroma virus, hence if doubly infected testes were kept for a few days in the cold before passing, most of the virus III died out, while the fibroma virus was hardly affected. Virus III was thus prevented from overwhelming the other.

In rabbit testes passed in this manner a common histological finding was an island of necrotic tissue in the middle of a fibromatous area. It could be seen that the necrosed tissue contained many hardly recognisable inclusions, while typical inclusions were present in almost every cell at its periphery. The focal character of such a virus III lesion was its outstanding feature.

The effect of virus III in accelerating the regression of fibroma lesions was best studied by inoculating the two viruses intradermally, both separately and in mixtures. In one such experiment dilutions of fibroma in saline were inoculated into one flank of a rabbit, into the other flank dilutions of fibroma in virus III suspensions were injected. The pure fibroma lesions persisted for 33 days, those of the mixture regressed after 14 days. In another similar test on two rabbits, control fibroma lesions were still present after 26 and 30 days respectively, while "mixture" lesions disappeared after 19 days.

An "interference phenomenon"

When mixtures of undiluted virus III and more dilute (1 : 100) fibroma virus were injected intradermally into rabbits, the virus III suppressed the fibroma completely. The flat erythematous lesions of virus III appeared about the third day at the site of inoculation of such mixtures and then disappeared within a few days. At control sites on the same rabbit inoculated with fibroma only, the thick characteristic fibroma nodules developed gradually and persisted for some weeks. Extracts of normal rabbit testis had no such action as virus III in suppressing the fibroma. Virus III could even inhibit the diluted fibroma when injected 4 hours after that virus, into the same marked site on the skin; when given 24 hours after the fibroma its suppressing action was less constant. When all the fibroma virus injected into a rabbit had been suppressed by mixing with virus III, that rabbit did not even develop an immunity to fibroma through escape of some virus to other parts of the animal. Yet the action of virus III was apparently purely local; injected elsewhere into a rabbit at the same time as a fibroma inoculation or 24 hours beforehand it produced no effect. We may guess that virus III acts by occupying the same cells as those affected by fibroma and crowding it out only by multiplying more rapidly. Its behaviour in the skin suffices to explain why its presence may interfere with the propagation of fibroma in the testis. Whether or not its inhibiting action has really close analogies with the phenomenon of "interference" between two viruses described in the literature is another matter. In the best known examples—the interference between two strains of yellow fever virus or between yellow fever and Rift valley fever viruses (*cf.* Hoskins, 1935; Findlay and MacCallum, 1937)—the effect is apparently a general and not a local one.

Separation of virus III from the mixture with fibroma

As already mentioned, a strain of fibroma free from virus III was obtained from stored glycerolated tissue. It was hoped that inoculation of this into rabbits would provide some fibroma-immune animals and that passage of the fibroma-with-inclusions through these would provide a pure strain of virus III. These hopes were not realised.

Pure fibroma virus inoculated intratesticularly into a rabbit immune to the same virus produces a mild inflammatory reaction, consisting chiefly of packing of the interstitial tissues with lymphocytes. This is doubtless an allergic reaction similar to that produced when virus III is injected into animals immune to virus III (Andrewes, 1928). On five occasions fibroma-with-inclusions material was inoculated into a fibroma-immune rabbit, but the

lesions obtained were only those of the allergic type, with, exceptionally, one or two intranuclear inclusions. Normal rabbits inoculated with the same material showed typical fibromatous proliferation with plentiful virus III inclusion bodies. When the mixture was passed successively through three fibroma immune rabbits in order to make sure of getting rid of all fibroma virus the virus III was lost also. There is no cross immunity between virus III and fibroma virus to explain these findings (Shope). Possibly the rabbits' allergic response to the fibroma created an environment unfavourable to the growth of virus III. More probably the fibroma lesions in the non immune rabbits produced a favourable nidus for a small amount of rather weak virus III and thus actually helped its survival.

Recourse was next had to tissue cultures. Virus III readily infects cultures of normal rabbit testis *in vitro* (Andrewes, 1929), but Miss Faulkner and I (Faulkner and Andrewes, 1935) failed to establish the OA strain of fibroma in this medium when we started with spun virus and normal testis tissue. Accordingly a centrifuged suspension of the testis of a rabbit infected with fibroma with inclusions was used to inoculate cultures in Carrel flasks containing minced normal rabbit testis and Tyrode's solution. After 4 days' incubation the supernatant fluid from these cultures was used to inoculate similar cultures. After a further four days these first subcultures were tested on rabbits and produced typical virus III lesions with inclusions and with no sign of fibroma histologically. Pieces of tissue from the primary culture and first subculture were also fixed and intranuclear inclusions were found in each case. With this method, then, it proved possible to separate virus III from the virus mixture.

The significance of the various findings described will be considered when attention has been paid to the association of virus III and a rabbit sarcoma.

VIRUS III AND TRANSPLANTABLE RABBIT SARCOMA RS 1

Since virus III can cause early regression of fibroma lesions, it was thought to be of interest to see whether it would similarly affect a true connective tissue neoplasm. Levaditi and Haber (1936) have shown that cell necrosis of mouse carcinomata may be induced by injecting them with fowl plague virus.

Rabbit sarcoma RS 1 (Andrewes and Ahlstrom) arose in a rabbit inoculated with both *vir* and fibroma virus. No fibroma virus can now be detected in the transplantable growth and its role in the aetiology of the tumour must be regarded as obscure. The tumour is a spindle celled sarcoma, frequently metastasising to lumbar lymph nodes and with a tendency to early central necrosis in the transplants. On attempting to infect RS 1 tumours with virus III,

A rabbit sarcoma, RS 1, was also found to have picked up and to be carrying virus III, as it was propagated by cell grafts. No evidence was obtained that virus III affects the growth of the sarcoma. The sarcoma could be freed from virus III by passage through one virus III-immune rabbit.

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BACTERIAL SPORES AS ANTIGENS

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(PLATE XXVIII)

A CONSIDERABLE amount of work has been done on the antigenic structure of bacterial flagella and somata and their relation to H and O types of agglutination. Much less attention has been paid to the antigenic possibilities of bacterial spores. Two opposite views have been put forward in the scanty literature on the subject: (1) that spores and bacilli each produce antibodies which are separate and distinct (Defalle, 1902; Mellon and Anderson, 1919) and (2) that spores produce no antibodies to themselves but give rise to H and O agglutinins for the bacillary forms (Krauskopf and McCoy, 1937). The evidence in support of these two views is not, however, in any way conclusive. The antisera which Defalle used had titres of rather a low order—1 : 120 and 1 : 170 for homologous spores and 1 : 40 and 1 : 20 for the corresponding bacilli. Mellon and Anderson treated their spore suspensions with 5 and 10 per cent. antiformin to remove bacilli—a procedure open to the objection that such treatment might alter the character of the spore antigen. The same may be said of the work of Krauskopf and McCoy, who also treated the spore antigen with 15 per cent. KOH to destroy bacillary forms but reached opposite conclusions. In view of the contradictory results of previously published work, the experiments described in this paper were carried out with five different organisms.

Is there a spore antigen?

To test this question a strain of *Cl. sporogenes* isolated from a haddock was employed which under anaerobic conditions at 37° C. grew and spored abundantly in a short time. Ten-day-old cultures from agar slopes were washed off in saline and injected into rabbits to prepare antisera. Films of the suspensions showed enormous numbers of spores and apparently few bacilli. No attempt was made to separate them. Six daily intravenous injections of 1.0 c.c. of suspensions containing approximately 5000 million spores per c.c. were given, followed by a week's rest and then by seven further daily intravenous injections of 1.0 c.c. The rabbit was bled on the fourth day after the last injection. At the same time H and O suspensions of bacilli were used to prepare corresponding bacillary antisera. From the results of agglutination tests with these sera (table I) it is evident that spores are antigenic. The spore antiserum contained agglutinins for both spores and bacilli. The titre for

bacilli was high—perhaps unexpectedly so considering the apparently small number of bacilli in the suspension used to prepare the antiserum—but the reason doubtless lay in the highly antigenic nature of the bacilli. This became apparent when the bacillary antisera were being prepared, since three intravenous injections on succeeding days of 1 c.c. of a suspension of H bacilli gave an antiserum with a titre of 1 : 300,000. Absorption tests were carried out with heavy suspensions of bacilli from cultures in 400 c.c. of 0.05 per cent. ascorbic acid broth grown for 18 hours at 42° C. to eliminate sporing as far as possible. The results (table I) showed that the H agglutinins

TABLE I
Agglutination of spores and bacilli of Cl. sporogenes

Test suspension	Spore antisera			Bacillary antisera	
	unabsorbed	absorbed		H	O
		with live bacilli	with spores		
Spores	10,240	10,240	40	Nil	Nil
H bacilli	300,000	40	300,000	300,000	20
O bacilli	640	Nil	640	40	10,240

could be reduced from a titre of 1 : 300,000 to one of 1 : 40 and the O agglutinins from one of 1 : 640 to nil, while the spore agglutinins remained unchanged at a titre of 1 : 10,240. Bacillary antisera (H as well as O) did not agglutinate suspensions of spores. In testing the bacillary antisera against the spore suspensions used to prepare the spore antiserum, it was found that small flocculent clumps of bacilli fell to the bottom of the tubes leaving unagglutinated spores in the supernatant fluid. This method of effecting mechanical separation of spores and bacilli proved useful in obtaining spore suspensions as free of bacilli as possible. With such suspensions no further agglutination took place even with undiluted bacillary antisera. Essentially similar results were obtained on several repetitions of the experiments. Thus while with *Cl. sporogenes* it had not proved possible to effect a complete separation of spore and bacillary antigens, it was none the less clear that both antigens existed and further, that the agglutinins to the two antigens could be separated by simple absorption. Similar results were obtained by applying the same methods to two other organisms—*B. cereus* of the National Collection of Type Cultures and a spore-bearing aerobic organism labelled "Soil I." Since the details merely confirm the above observations without adding data of new significance they have not been quoted.

It still remained possible that spores might give rise to bacillary as well as to spore agglutinins, but the experiments with another organism, reported below, appear to be against this hypothesis.

Do spores give rise to bacillary agglutinins?

Much of the controversy noted in the literature on this question arises from the difficulty of obtaining spores free from bacilli and in the methods chosen to attain that end. In the course of the examination of sporing organisms from different sources on different media we were fortunate to meet an organism which appeared to lend itself admirably to the purposes of this investigation. This was a Gram-positive spore-bearing aerobic organism obtained from garden soil, to which it has not so far been possible to give a name, although it has certain characters like those of *B. cereus*, with which it has been compared.

The organism produced confluent whitish yellow growth on agar slopes at 22°, 30° or 37° C., optimum growth being obtained at 30° C. Separate colonies were raised, circular and opaque. Gelatin was not liquefied; Loeffler's (sheep) serum slopes were liquefied completely in 24 hours at 37° C. Acid was produced in glucose, lactose, sucrose and mannitol but not in dulcitol. Indole was not produced in peptone water. Growth on potato was white and slimy and a similar growth occurred at first on egg medium followed by liquefaction and yellowing. Glucose agar cultures produced a very mucoid type of growth; in broth there was a deposit but no surface scum. Fluid cultures were motile. A similar organism has since been obtained from a specimen of antral lavage. *B. cereus*, which the organism resembles in some features, does not, however, conform in other points and, in particular, it does not yield spore suspensions so free from bacilli as does the organism in question, which has meantime been labelled "Soil 2."

The important feature of the organism is that on asparaginate agar* plates it spores rapidly and completely. In two days at 37° C. spores were abundant and bacilli already much reduced in number, while at the end of a week very few bacilli could be demonstrated. After 10 days only a few rather swollen bacillary forms could be identified and free spores were present without any evident bacillary protoplasm attached (fig. 1).

The surface growth of a heavily inoculated asparaginate agar plate incubated for 10 days at 30° C. was accordingly used to make a spore antiserum. The growth was washed three times in 10 c.c. saline and four intravenous injections of 1 c.c. were given on successive days to a rabbit which was bled ten days after the last injection. A suspension of bacilli from a 16-hour culture

* Formula of sodium asparaginate agar

Distilled water	1000 c.c.	NH ₄ H ₂ PO ₄	1.3 g.
Agar	20 g.	CaCl ₂	0.1 "
Sodium asparaginate	1 "	KCl	0.1 "
Glucose	1 "	FeCl ₃	a trace
MgSO ₄ · 7H ₂ O	0.2 "		

on an agar slope in 0.2 per cent. formol-saline was used to prepare an H anti-serum. Agglutination tests were carried out between the two sera and H and O suspensions of bacilli and spore suspensions prepared as above.

The results (table II) were perfectly clean-cut. Spore antiserum agglutinated homologous spores to a titre of 1 : 1280 but did not agglutinate H or O suspensions of bacilli, either in dilutions as low as 1 : 20 in the water-bath or undiluted upon slides. Similarly H

TABLE II
Agglutination of spores and bacilli of organism "Soil 2"

Antiserum	Suspension	Titre
Spore	Spores	1280
	H bacilli	Nil
	O bacilli	"
Bacillary (H)	Spores	Nil
	H bacilli	2560

antiserum agglutinated H suspensions of bacilli to a titre of 1 : 2560 but did not agglutinate spore suspensions. The clean-cut nature of the results can be illustrated by means of stained preparations of slide agglutinations (figs. 2 and 3).

A mixed suspension of spores and bacilli is agglutinated with spore antiserum on one part of the slide and with bacillary antiserum on another. Agglutination occurs at once. The slide is allowed to dry, fixed and stained with carbol-fuchsin-methylene blue spore stain in the usual way.* It will be seen that the spore antiserum agglutinates only the spores of the mixed suspension (fig. 2) while the bacillary antiserum agglutinates only the bacilli (fig. 3).

In view of these results it was decided to study the transition on an asparaginate agar plate from bacilli to spores and to see whether the morphological and serological changes could be correlated. An agar culture was also studied as control. The results are summarised in table III and are shown for the asparaginate agar culture in fig. 4. It will be seen that the serological and morphological changes were closely related. A second spore antiserum prepared in the same manner agglutinated spores to a titre of 1 : 20,000 and suspensions of live bacilli from agar slopes incubated overnight to a titre of only 1 : 80.

* *Spore stain used*

Strong carbol-fuchsin, steaming, 1.3 minutes.

Wash in water.

1 per cent. sulphuric acid, 1 minute.

Wash in water.

Wash in spirit for about 10 seconds.

Counterstain with watery methylene blue for 2.5 minutes.

SPORES AS ANTIGENS



FIG. 1.—Organism "Soil 2."
Free spores without bacilli.
Ten-day culture on aspara-
ginate agar at 30° C. Spore
stain. $\times 2500$.

FIG. 2.—Organism "Soil 2."
Mixed suspension of spores
and bacilli with spore anti-
serum. Spores agglutinated;
bacilli not agglutinated.
Spore stain. $\times 470$.



FIG. 3.—Organism "Soil 2." Mixed suspension of
spores and bacilli with
bacillary antiserum. Bacilli
agglutinated; spores not
agglutinated. Spore stain.
 $\times 470$.

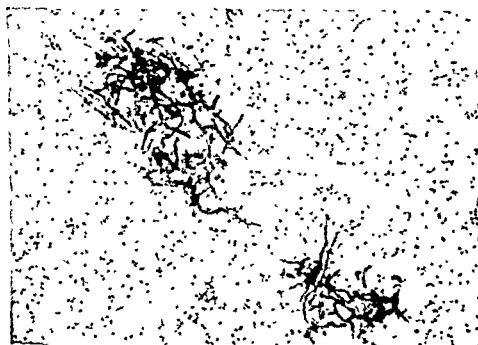


TABLE III

Agglutination of culture of organism "Soil 2" in relation to morphology

	Hours	Morphology	Agglutination with bacillary serum	Agglutination with spore serum
Agar culture	18	Bacilli; no spores	1 : 2560	No agglutination " 1 : 20 "
	42	Bacilli; occasional spore	1 : 1280	
	66	Bacilli; few spores	1 : 1280	
	90	Bacilli; few spores	1 : 1280	
Asparaginate culture	18	Bacilli; few attached spores	1 : 1280	1 : 120
	42	Spores outnumber bacilli	1 : 40	1 : 1280
	66	Spores; few bacilli	1 : 40	1 : 1280
	90	Spores; very few bacilli	1 : 20	1 : 2560

Readings after 2 and 4 hours in the water-bath at 56° C.

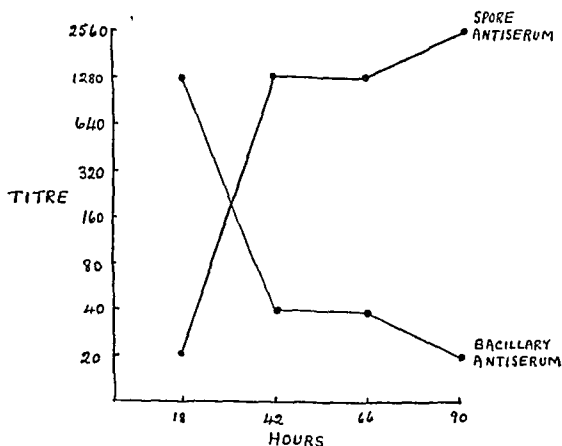


FIG. 4.—Agglutination of culture of sporing organism "Soil 2" at different stages of growth on asparaginate agar.

It has been suggested (Krauskopf and McCoy) that spores can stimulate the production of antibodies for the bacilli to which they will give rise upon germination and that no antibody is formed against the spores themselves. The results quoted above do not support such conclusions and indeed lead to the view that spores are antigenic and that the spore antigen is separate and distinct from the corresponding bacillary antigens. Two additional experiments may be quoted which are consistent with this view. If spore and bacillary antigens are separate and distinct entities

germination must be regarded as a process which results in the transition from one antigenic phase to another rather than as the simple emergence of an antigenically unaltered bacillus from a protective spore case. That some alteration in the chemical nature of spores does occur in the earliest stages of germination has been shown by making stained preparations at intervals of five minutes of heavy spore suspensions of organism "Soil 2" in broth incubated at 37° C. All the preparations were made and fixed upon one slide and stained as described above. In the preparation before incubation the majority of the spores were pale red while a few took on a bright red colour. After five minutes' incubation a small number of dark red spores appeared in the film; in ten minutes their number had increased, and a few purple stained spores were seen. The number of purple spores continued to increase; many of these took on a swollen irregular shape and after 1 hour bacilli could be recognised. The change in staining reaction, however, was noted before any change in shape had occurred and may be put forward as evidence in support of the hypothesis that a chemical (*i.e.* antigenic) change occurs in the spores during the process of germination. The changes in staining reaction did not occur in spores suspended in broth and kept at 60° C. or in the ice chest, even overnight, *i.e.* in conditions in which they do not germinate. Similar changes were found to occur during the germination of the spores of *B. mesentericus*.

Additional evidence in favour of the separate and distinct nature of spore and bacillary antigens has been obtained by a study of the antigenic properties *in vivo* and *in vitro* of autoclaved spore suspensions of *B. mesentericus*. The suspensions were like those of *Cl. sporogenes* in that they contained a great predominance of spores but a few bacilli remained.

A strain of *B. mesentericus* from the National Collection of Type Cultures was grown for 8 days at 37° C. on asparaginate agar. At the end of that time films showed that it was almost completely spored but a few chains of bacilli could still be found. The growth from the surface of 6 plates was suspended and washed three times in normal saline and divided into two portions of 5 c.c. The first portion was autoclaved for 20 minutes at 15 lb. and thereafter stored in the ice chest. The second was stored in the ice chest without being autoclaved and is referred to as "live spores." Antisera were prepared by injecting rabbits with the autoclaved and live spores. In each case 4 injections of 1 c.c. were given intravenously on successive days and the animals bled on the thirteenth day after the last injection. At the same time a bacillary antiserum was prepared from the washed deposit of a young broth culture of the same strain. Three injections of 1 c.c. were given intravenously on successive days and the rabbit bled on the tenth day after the last injection. A fresh culture was used each day in order to reduce the number of spores as far as possible. Stained films of the centrifuged deposits confirmed the fact that their number was very small—not more than one in every three 1/12th in. oil immersion fields with a thickly spread smear.

The results of agglutination tests with the three antigens (1) live spores, (2) autoclaved spores and (3) live bacilli and their corresponding antisera are shown in table IV. It is evident that the

TABLE IV
Agglutination of spores and bacilli of B. mesentericus

Antiserum	Suspension	Titre
Live spore	Live spores	20,480
	Autoclaved spores	5120
	Live bacilli	1280
Autoclaved spore	Live spores	10,240
	Autoclaved spores	2560
	Live bacilli	Nil
Bacillary	Live spores	80
	Autoclaved spores	20
	Live bacilli	120

process of autoclaving the spores had not significantly altered their antigenic activity either *in vivo* or *in vitro*. On the other hand, the antigenic activity *in vivo* of the bacilli mixed with the spore suspension was completely destroyed by autoclaving. Similar conclusions were stated by Defalle, although they were based on less adequate data in view of the low titre sera employed. The evidence in this experiment is additional support for the view that the antigenic properties of spores are different from those of the corresponding bacilli.

Summary

The experiments reported in this paper were carried out with five different organisms—*Cl. sporogenes*, *B. cereus*, *B. mesentericus* and two aerobic spore bearing organisms from soil which in some ways resemble *B. cereus* although they have not all the characters of this organism. With all of them it was possible to produce spore antisera which would agglutinate spore suspensions of the corresponding organism. Experiments were carried out along three different lines to see how far it was possible to separate the spore and bacillary antigens and their related antibodies.

1. *By agglutinin absorption.* Antisera against *Cl. sporogenes*, *B. cereus* and the organism "Soil 1" were prepared by injecting suspensions largely composed of spores but in which small numbers of bacilli were also present. These antisera contained agglutinins against both spores and bacilli but by absorption with heavy suspensions of the appropriate antigen it was possible to remove one agglutinin without appreciably lowering the titre of the other.

2. *By growth on special medium.* The organism "Soil 2" when grown for 10 days on sodium asparaginate agar produced growths containing spores and so few bacilli that the latter were unable to stimulate antibody formation. By this means a spore antiserum was obtained which agglutinated spores but not bacilli, thus proving that spores do not give rise to bacillary agglutinins although they are antigenic.

3. *By autoclaving spore suspensions.* Antisera were prepared against autoclaved and unautoclaved suspensions of *B. mesentericus* containing few bacillary forms. Autoclaving destroyed the antigenic activity of the bacilli but did not significantly alter the antigenic properties of the spores.

Conclusions

(1) Bacterial spores are antigenic.

(2) Spore antigen is separate and distinct from the antigens of the bacilli to which the spores give rise on germination.

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INFECTION OF WHITE MICE WITH THE THREE TYPES OF *C. DIPHTHERIÆ*

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INFORMATION about the possibility of infecting white mice with diphtheria bacilli is scanty and to some extent contradictory. The opinion expressed in many text books that mice cannot be infected is apparently due to Loeffler's remark in his description of the properties of the diphtheria bacillus. The findings of Roux and Yersin and of Behring and Kitashima (quoted by Kolle and Schlossberger, 1919a) showed, however, that diphtheria toxin is able to kill white mice if given in very big doses—80 M L D for guinea

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serum was sometimes sufficient to kill and *C. diphtheriæ* could nearly always be recovered from the spleen. They concluded that a general infection occurred. Toxins of the same strains treated with toluol did not kill mice in doses equivalent to 5 100 M L D for guinea pigs. After showing that there was no true diphtheria antitoxin in normal mouse blood they successfully treated mice with antitoxic serum, both prophylactically and therapeutically. Hippke (1923), however, only occasionally succeeded in producing fatal infection in mice with cultures of *C. diphtheriæ*. Out of 15 strains tested on 103 mice, only 6 proved pathogenic and only 14 mice died, although a certain number showed signs of illness while recovering later. Wolff (1922) also showed that only "suitable" strains are able to cause infection and death and was able to reproduce the effect of living cultures by injecting repeated doses of toxin treated with toluol. Lately Kildyshewa and Potapchuk (1937) have shown that it is possible to establish the pathogenicity of diphtheria strains in mice.

This survey shows that the findings of Kolle and Schlossberger have been only partially confirmed as far as the general pathogenicity of diphtheria strains for white mice is concerned. The reasons for this might be (a) differences in dosage, (b) differences in the strains of mice used or (c) differences in the strains of *C. diphtheriæ*. The last explanation has become more plausible since McLeod and his collaborators (Anderson *et al.*, 1931) established the existence of three types and their different pathogenicity for man. There is a possible analogy in the findings of Dimitrijević Speth and Arsenjević (1937), who showed that spermophils exhibited varying resistance to infection with the three types of *C. diphtheriæ*.

Investigations were therefore carried out to determine whether there are any differences between the *gravis*, *mitis* and *intermedius* types of *C. diphtheriæ* in their pathogenicity for white mice and whether a general infection occurs. Consideration was also given to the question whether any change of type or change of diphtheria bacilli into diphtheroids takes place within the body of white mice, as claimed by Preuner (1936).

Pathogenicity of the three types of C. diphtheriæ for white mice

As previous unpublished experiments of my own had shown that intravenous or intraperitoneal injection resulted in a speedier and more constant infection of white mice than subcutaneous injection, only these two methods were employed. Young mice 6-8 weeks old, usually in groups of three or more for each strain, were injected with 1 c.c. of a suspension in peptone water or saline of a 24-hour Loeffler culture standardised to the opacity of a standard suspension of roughly 1500 million organisms per c.c. The viable counts of these suspensions on glucose agar plates varied widely, from 15 to 375 million per c.c., but in most cases there were about 150 million. The ratio of viable to total bacteria judged by opacity varied from experiment to experiment and to about the same extent with all types. This variation must have been due to the presence of unusually large proportions of dead organisms in the Loeffler culture or to the unsuitability of the agar sample used. In order to eliminate the possibility of an action of diphtheria toxin produced in the cultures on Loeffler's medium, 14 strains were washed twice in peptone water or di-stilled water before being made up to the final suspension. Such treatment made no difference in the result.

In all, 112 mice were inoculated with 36 strains, of which the types and pathogenicity for the guinea-pig had previously been established. Ten were of the *gravis* type, 10 of the *mitis* and 16 of the *intermedius*. All had recently been isolated from fresh cases and most of them were obtained in the Leeds area. Professor H. D. Wright generously sent 6 *intermedius* strains from Liverpool, Professor H. B. Maitland 1 from Manchester and Dr W. M. Elliott 2 *intermedius*, 1 *mitis* and 2 *gravis* strains from Glasgow. Five *gravis*, 5 *mitis* and 6 *intermedius* strains were injected intravenously and 5 *gravis*, 5 *mitis* and 10 *intermedius* intraperitoneally. Intraperitoneal injection was used to obviate the difficulty of determining the significance of the presence of *C. diphtheriæ* in blood from the tail after an intravenous injection. It seemed possible that the occasional positive findings might be erroneous and due to contamination from cellulitis of the tissues of the tail. As the finding was one to be established beyond reasonable doubt it seemed desirable to adopt a method which eliminated the possibility of this sort of error. No noteworthy differences in results and post-mortem findings were observed between the two methods except for a general or localised peritonitis after intraperitoneal injection. If death did not occur at an earlier date the mice remained under observation until the end of the fourth week after injection.

Table I shows the general results. On the basis of the average fatality rate the *mitis* type is the most lethal (93.3 per cent.), the *gravis* next (68.6) and the *intermedius* least (46.8). Death occurred in from 20 hours to 24 days after infection, the animals infected with the *mitis* type dying sooner than those infected with *gravis* or *intermedius*. Most of the *intermedius* strains killed one or two

TABLE I—The virulence of different types of diphtheria bacilli for white mice

Strain	Number of mice injected	Route	Died	Survived but ill	Survived without illness
Gravis					
Wilk	6	Intravenous	4	1	0
Hewson	6	"	6	1	0
Nasw	3	"	2	0	1
Mayrshill	3	"	3	0	0
SS 190	2	"	0	0	2
436	3	Intraperitoneal	2	0	1
Gale	3	"	3	0	0
McLaugh	3	"	3	0	0
18	3	"	0	1	2
7	3	"	1	2	0
	35		24=68.6 per cent	5=14.3 per cent	6=17.1 per cent
				31.4 per cent	
Mitis					
29	3	Intravenous	3	0	0
60 M	3	"	2	0	1
64	3	"	3	0	0
489	3	"	3	0	0
1022	3	"	2	0	1
376	3	Intraperitoneal	3	0	0
437	3	"	3	0	0
McCat	3	"	3	0	0
443	3	"	3	0	0
442	3	"	3	0	0
	30		28=93.3 per cent	0	2=6.7 per cent
Intermedius					
930	3	Intravenous	0	0	3
FC 172	3	"	1	0	2
E 140	3	"	2	0	1
2110	3	"	0	0	3
7157	3	"	0	1	2
7177	2	"	1	0	1
358	3	Intraperitoneal	3	0	0
D 14428	3	"	0	0	3
7171	3	"	1	1	1
McCarr	3	"	1	0	2
608/3	3	"	3	0	0
4064	3	"	2	0	1
60 I	3	"	1	0	2
4537	3	"	1	1	1
Lees	3	"	3	0	0
20	3	"	3	0	0
	47		22=46.8 per cent	3=6.4 per cent	22=46.8 per cent
				53.3 per cent	

Mitis intermedius = D/SE > 4*Mitis gravis* = D/SE > 2*Gravis intermedius* = D/SE < 2

out of three mice, while 4 strains killed all mice and 4 caused no deaths at all.

The M.L.D. of the *intermedius* strains which had killed all the mice infected was perhaps higher than that of similarly active *gravis* or *mitis* strains. In each of five experiments one such *intermedius* strain was compared with one selected *gravis* or *mitis* strain, four mice being infected with 1500, 1000, 500 and 150 million organisms of each, determined by opacity. In four of these experiments viable counts were made. Varying results were obtained, the number of organisms ranging from 15 to 150 million. In each experiment the viable count was about equal for both strains used. With the *intermedius* strains only the highest dose—1500 million—killed, while with *mitis* and *gravis* strains the dose of 1000 million also killed. Although the animal groups used in these experiments are too small to draw any definite conclusions, the 5 *intermedius* strains, selected as the most virulent strains of this type, seemed to have a higher M.L.D. than corresponding *gravis* or *mitis* strains. These findings correspond to those of Dimitrijević-Speth and Arsenijević for spermophils, which are more resistant against the *intermedius* type.

Of the surviving mice a certain percentage in the *gravis* and *intermedius* groups showed signs of more or less severe illness (*gravis* group 14·3 per cent.; *intermedius* group 6·4 per cent.). The severity of the illness varied from a roughening of the coat or one or two days' diarrhoea to marked disturbances of the sense of balance, unrest, paralysis and similar symptoms of a cerebral nature. Loss of weight and stupor were also common. All these symptoms were also observed in one or other of the mice which died. Some of the *mitis* strains caused paralysis of all extremities which appeared 24-48 hours after infection and spread quickly over the whole body. This condition continued for several days until the respiration became gradually slower and the animals died, apparently of paralysis of the respiratory centre. Similar conditions were also observed in a few of the mice infected with the two other types.

One feature of the post-mortem examination was the intense red colour of the suprarenal glands. In animals which died later than a fortnight after injection the suprarenals were slightly pink or yellow in appearance. Often intense hyperæmia and even hæmorrhages were observed in the subcutaneous tissue of the abdominal wall, especially in the vicinity of lymphatic glands, which were swollen, red and sometimes purulent. The intestines were occasionally hyperæmic as were also the other abdominal organs if the infection was not a protracted one. If the infection was more or less subacute or chronic, nearly all the fat of the subcutaneous tissue and organs disappeared and in consequence the liver appeared very dark in colour. In six instances the kidneys

were very pale. Wolff found this to be a sign of nephrosis with necrosis and degeneration of the tubular epithelium. Two of the strains causing pale kidneys were of the *gravis* type, the other four being *intermedius*. Only in two of these cases were diphtheria bacilli—1 *gravis* and 1 *intermedius*—recovered from the kidneys. Four strains—2 *intermedius*, 1 *gravis* and 1 *mitis*—caused hæmorrhages in the meninges so severe that when the skin was removed the whole skull appeared dark red. Diphtheria bacilli could not be grown from the meninges or from the brain.

Generalisation of infection

In order to obtain an idea of the frequency and extent of generalised infection tellurite plates were inoculated directly from organs and fluids *post mortem*. This was done as soon as possible and in most cases immediately after death, with all aseptic precautions. The skin over the abdominal wall and chest was removed after moistening with alcohol which was burned off. The muscular abdominal wall was treated in the same way and then the peritoneum was opened. After the whole surface had been seared with a hot iron, each organ was cut with sterile instruments and the surface of the section smeared on the surface of a tellurite plate. Peritoneal and pleural fluid as well as heart blood were aspirated into Pasteur pipettes and one or more drops plated out with a loop. Sterile access to the brain was similarly secured, the skull being seared directly with a Bunsen flame. A loop was put into the substance of the cerebrum and cerebellum and turned several times in order to get contact with as much tissue as possible, and a loopful of brain tissue plated out. Peritoneal fluid, spleen, kidneys, liver, pleural fluid, heart blood, lungs and brain were investigated. The method possibly erred on the side of caution, as organisms might have been killed by the repeated application of heat to such small surfaces. In this way the failure to grow diphtheria bacilli from the peritoneal exudate in spite of an existing peritonitis might be explained.

Table II (p. 248) gives a general survey of these experiments. It is evident that a generalisation of infection had occurred. Ample evidence of this was obtained in positive cultures from the heart and brain in a number of cases apart from similar findings in organs of the abdominal cavity which might possibly be explained by incomplete sterilisation of the surface of the viscera. A feature in table II is that with the exception of the peritoneal fluid all figures for the *intermedius* group are far below those for the *gravis* and *mitis* groups and that those for the *mitis* group are the highest. The fact that growth could be obtained from brain tissue, especially in the *mitis* group, seems to suggest that at least part of the cerebral symptoms might be due to the presence of *C. diphtheriæ*, with local production of toxin.

Evidence of septicæmia was also seen in a histological section of the liver of a mouse which died 13 days after intravenous infection with a *gravis* strain. There were Gram positive bacilli occurring singly and in pairs and situated intracellularly, and a small abscess

TABLE II.—The recovery of diphtheria from white mice after intravenous and intraperitoneal injection

Strain	Site of Injection	Number of mice died	Number of mice in which diphtheria bacilli were recovered from								
			some organ	peritoneal fluid	spleen	kidney	liver	pleural fluid	heart blood	lung	brain
Gravis											
Wilk.	Intravenous	1	1	0	3	1	3	3	3	2 (1-)*	2 (3-)
Hew.	"	6	1	1	3	1	1	3	3	1	1
Naw.	"	2	2	1	1	2	2	2	2	1	0
Mayfield	"	3	3	2	2	3	3	2	2	0	0
L36	Intraperitoneal	2	2	0	2	2	2	2	2	0	0
Gale	"	3	3	3	3	3	3	3	3	1	0
McLaugh.	"	3	3	3	3	3	3	3	3	1	0
7	"	1	1	1	1	1	1	1	1	1	0
Number		21	22	9	10	21	19	13	11	12 (1-)	1 (3-)
Per cent.		.	91.7	37.5	66.7	87.5	79.2	51.2	58.3	52.1	19.0
Mittis											
29	Intravenous	3	3	1	2	2	2	2	2	3	1
60 M	"	3	3	0	2	2	2	2	2	3	0
61	"	3	3	3	3	3	3	3	3	1	0 (1-)
180	"	3	3	3	3	3	3	3	3	1	0
1022	"	3	3	1	2	2	2	2	2	1	0
370	Intraperitoneal	3	3	1	3	3	3	3	3	2	3
137	"	3	3	3	3	3	3	3	3	1	0
McGat.	"	3	3	3	3	3	3	3	3	1	0
143	"	3	3	3	3	3	3	3	3	1	0
142	"	3	3	3	3	3	3	3	3	2	2
Number		23	28	18	25	26	27	24	22	20	11 (1-)
Per cent.		.	100	61.3	59.3	92.9	96.4	85.7	78.6	71.4	19.7
Intermedius											
PC 172	Intravenous	1	0	0	0	0	0	0	0	0	0
PC 110	"	2	0	0	0	0	0	0	0	0	0
7177	"	1	0	0	0	0	0	0	0	0	0
358	Intraperitoneal	3	3	3	1	1	3	1	1	0	0
7171	"	1	1	1	1	1	1	0	0	0	0
McGarr	"	1	0	0	0	0	0	0	0	0	0
608/3	"	3	3	3	2	1	2	1	0	0	0
1004	"	3	3	3	0	1	1	1	1	0	0
60 I	"	1	1	1	1	1	1	0	0	0	0
4577	"	1	1	1	1	1	1	0	0	0	0
L265	"	3	3	2	0	2	2	0	0	0	0
20	"	3	3	3	3	1	3	0	2	0	0
Number		22	17	15	9	9	15	4	1	1	1
Per cent.		.	77.3	68.2	40.9	40.9	68.2	18.2	18.2	15	15

* (1—), (2—) etc., = organ not examined for *C. diphtheriae* in 1, 2 etc. cases.

containing numerous organisms with all the characteristics of *C. diphtheriæ*.

An attempt was also made to demonstrate the generalised infection during life. One to three days after intraperitoneal infection 1 or 2 drops of blood, taken by cutting off the tip of the tail after cleaning it with alcohol, were inoculated on tellurite plates. This was done in 45 mice with all types of *C. diphtheriæ*. In 24 cases the result was negative but in 21 cases one or other of all three types of *C. diphtheriæ* was grown from the tail blood. In order to prove this point beyond doubt 8 *mitis* strains were each injected into 3 mice. One day after injection the tails were cleaned with alcohol, allowed to dry and after moistening with broth smeared upon the surface of a chocolate and a tellurite plate. Then the tips of the tails were cut off and a chocolate and a tellurite plate inoculated with the tail blood. The *mitis* strains were recovered from the tail blood of all but two of the mice but not from the surface of the tail.

Constancy of type in mouse passage

In every case where *C. diphtheriæ* was grown from organs or tail blood a full examination was carried out to determine the type of organism recovered. In every case they had exactly the same properties as the strain injected. Growth of diphtheroids was found in only a very few cases where the autopsy had been unavoidably delayed. In these cases there was always a growth of streptococci besides diphtheroids and *C. diphtheriæ*.

Discussion

The figures in table I suggest that there is a difference in the pathogenicity of the three types of *C. diphtheriæ* for white mice. A dose of 1500 million organisms, determined by opacity, seems to be about the M.L.D. for *intermedius* strains, whereas for *gravis* and *mitis* strains it is possibly lower. According to these findings the *mitis* type in mice seems to take the place of *gravis* in man, *gravis* in mice might be compared with *intermedius* in man and the *intermedius* type has the lowest pathogenicity for mice as has *mitis* for human beings. It is possible that all strains investigated by Kolle and Schlossberger belonged to the *mitis* type, while Hippke and Wolff used strains of another type. The results in table II are in accord with the view of Kolle and Schlossberger, that diphtheria bacilli cause a generalised infection in white mice. In my observations *C. diphtheriæ* has no predilection for any particular organ. The *intermedius* type cannot be found so frequently in internal organs as the other two types. This again suggests that there is an inversion of pathogenicity, pathogenic action and capacity of invasion of the various types for mice as compared with human

beings (Clauberg and Plenge, 1937) and guinea-pigs (Robinson and Marshall, 1934; Gundel and Erzin, 1936; Zinnemann and Zinnemann, 1939), in which the *gravis* type is found more frequently in the internal organs than the *mitis*. It is the *gravis* type which, according to McLeod, Orr and Woodcock (1939), produces the more intense pathological lesions in men.

Summary

1. It is possible to produce a fatal infection of white mice with all three types of *C. diphtheriæ*.

2. The findings suggest that for mice the *mitis* type is the most and the *intermedius* the least pathogenic, the *gravis* type occupying an intermediate position.

3. *C. diphtheriæ* can cause a septicæmia in white mice, the order of invasiveness being *mitis* > *gravis* > *intermedius*.

4. No alteration was found in any of the properties of the three types of *C. diphtheriæ* as a result of passage through white mice.

I wish to express my deep indebtedness to Professor J. W. McLeod for his guidance and advice in this work and to Professors H. B. Maitland and H. D. Wright and Dr W. M. Elliott for strains of *C. diphtheriæ*.

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BLOOD CHANGES AND POST-MORTEM FINDINGS FOLLOWING INTRAVENOUS INOCULATION OF SHEEP WITH CULTURE FILTRATES OF *CL.* *WELCHII*, TYPES A, C AND D

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THE information available in regard to the intra-vitam effects of *Cl. welchii* toxin on the blood refers mainly to its hæmolytic action. Further, the clinical and experimental observations made deal with the toxæmia caused by *Cl. welchii* of classical gas gangrene, very little attention having been paid to the toxins formed by other members of the group. The literature relating to the classification of the different types of *Cl. welchii* has recently been reviewed by Dalling and Ross (1938) and McCoy and McClung (1938).

The object of the present paper is to record and compare the symptoms, changes in the chemical and physical properties of the blood and post-mortem findings following the intravenous inoculation of sheep with culture filtrates of *Cl. welchii* of types A, C and D in Wilsdon's classification (1931), and evidence will be presented to show that, while no characteristic blood picture follows the injection of type C toxin, type A produces a marked hæmolytic anæmia and type D a typical anhydræmia. Minor differences in symptoms, blood picture and post-mortem findings are also recorded.

In the course of a bacteriological investigation of grass sickness in horses, the toxin of *Cl. welchii* type D was detected in filtrates prepared from the contents of the small intestine of a small proportion of horses affected with the peracute or acute form of the disease (Gordon, 1934). About the same time Dr Henry Dryerre (unpublished experiments) detected a marked and progressive rise in the hæmoglobin and sugar content of the blood of affected horses, together with a slight fall in the blood calcium. Massive doses of a culture of *Cl. welchii* type D were therefore given by the mouth to three horses, which died after showing similar blood changes.

It was observed by one of us (J. S.) that urine from cases of "pulpy kidney disease" in sheep (an entero-toxæmia caused by the toxin of *Cl. welchii* type D) contained a great deal of sugar unaccompanied by albumin.

This suggested that in this type of toxæmia the toxin must affect the normal sugar metabolism so as to raise the blood sugar above the threshold value. Blood samples from a few cases showed that the sugar content was indeed high, in some cases over 350 mg. compared with the normal 70-80 mg. per 100 c.c. This upset of sugar metabolism could not be detected in animals affected with lamb dysentery or with "struck"—entero-toxæmias caused by the toxins of *Cl. welchii* types B and C respectively.

The severe anæmia which characterises certain cases of gas gangrene infection in man has been noted by a number of investigators. Klotz and Holman (1911) found it to be an outstanding feature in a series of infections occurring in coal mines. Bull and Pritchett (1917), who first demonstrated the existence of the soluble toxin *in vitro*, recognised its hæmolytic properties. It has been shown (Report on the anaerobic infections of wounds, 1919) that filtrates of *Cl. welchii* of classical gas gangrene are hæmolytic both *in vitro* and *in vivo*, and produce necrosis of skin and muscle and marked œdema of the subcutaneous tissues. The hæmolytic action *in vivo* is best seen after intravenous inoculation, though it can also be demonstrated constantly in mice which die after intramuscular injection. Bull and Pritchett relate the case of a rabbit which, prior to a dose of 1.0 c.c. of toxin intravenously, had a red cell count of 5.4 millions. Seven hours after inoculation the count was only 1.0 million per c.mm. In a pigeon an initial count of 4.28 million red cells fell 27½ hours after inoculation to 0.8 million. Henry (1922) records that rapidly progressive anæmia was a familiar feature of gas gangrene during the European War. Blood counts a few hours before death invariably showed a serious depletion in the number of red cells and counts of less than one million per c.mm. were not infrequent in rapidly developing cases.

Chamberlin (1933), in acute cases of enzootic toxæmic jaundice—a disease of sheep and cattle in Australia, sudden in its onset and characterised by anæmia, intense icterus and hæmoglobinuria—records red corpuscle counts of 0.8-3.5 million as compared with a normal count (Norris and Chamberlin, 1929) of 11-12 million. Rose and Edgar (1936) conclude that this disease is essentially an entero-toxæmia due to *Cl. welchii* type A and have renamed it entero-toxæmic jaundice of sheep and cattle.

Thus there is evidence of the occurrence of acute hæmolytic anæmia in the toxæmia caused by *Cl. welchii* type A, whereas the observations of the biochemists at this Institute, referred to above, suggest that in the toxæmia due to type D there is a progressive rise in the hæmoglobin content of the blood.

METHODS

Origin, preparation and typing of filtrates

Types A and C filtrates were prepared from strains N.C.T.C. 26 and W. 49 obtained from the Wellcome Physiological Research Laboratories, type D from a culture, H. 35/4, isolated at this Institute from the contents of the small intestine of a horse which had died of acute grass sickness.

Cultures were grown in meat broth (pH 7.6) prepared from horse fleah. Types A and C were incubated for 11 hours, type D for 6 days at 37° C. The cultures were examined for purity, clarified by filtration through paper pulp and passed through a British Berkefeld candle. The A and C filtrates were precipitated by the addition of 70 per cent. ammonium sulphate and the precipitate was dried *in vacuo* over calcium chloride. The dry stable

product was reconstituted for use by dissolving 100 mg in 50 c.c. of saline. The type D filtrate was retained in the liquid state and its toxicity for mice remained constant throughout. The filtrates are hereafter referred to as toxins.

For each toxin the M.L.D. was determined in mice intravenously and the M.R.D. in guinea pigs intradermally in a large number of animals over a period of several months as well as the M.H.D. *in vitro*, i.e. the amount required to produce 50 per cent. hemolysis in a 6 per cent. saline suspension of sheep red cells. The results are recorded in table I.

TABLE I
Result of titration of toxins

Type of toxin	M.L.D. in mice I.V. (mg.)	M.R.D. in guinea pigs I.D. (mg.)	M.H.D. <i>in vitro</i> with 6 per cent. sheep red cells in saline (mg.)
A	0.6	0.18	0.08
C	0.02	0.016	0.14
D	0.004	0.01	0.40

The toxins were typed by mixing them with different antitoxins *in vitro* and, after contact for one hour, inoculating mice intravenously from each mixture. The antisera used were type A (G.G. 3269) and type C (R. 3589), prepared in horses and kindly supplied by the Wellcome Physiological Research Laboratories, and type D (H. 35/4) prepared in a horse at this laboratory from culture H. 35/4. Each filtrate behaved as an orthodox type according to Wilsdon's classification. Thus 0.3 c.c. of type A toxin was neutralised by 0.001 c.c. of type A serum and by 0.1 c.c. of type C and 0.01 c.c. of type D serum. Type C toxin in a dose of 0.01 c.c. was neutralised by 0.001 c.c. of type C serum but not by 0.1 c.c. of types A and D. Type D toxin in a dose of 0.002 c.c. was neutralised by 0.0009 c.c. of type D serum but not by 0.1 c.c. of types A and C serum.

Biochemical methods

Sugar was estimated by the Folin and Wu colorimetric method, non protein nitrogen by a Nesslerisation method, hemoglobin by the Newcomer disc method, creatinine by the picric acid sodium hydroxide method, amino acid nitrogen by the Folin and Wu naphthoquinone sulphonic acid method, calcium by the Kramer and Tisdall method, magnesium by a modification of the Denis method, phosphates by the Fiske and Subbarrow methods, chloride by Whitehorn's variation of Volhard's method, and potassium by the cobaltinitrite precipitation and permanganate titration method.

Examination of the blood

The coagulation time, erythrocyte count, total and differential leucocyte counts, platelet count, fragility of red cells, specific gravity, corpuscular volume and sedimentation rate were determined. The technique is recorded in more detail by Stewart and Holman (1940) and was briefly as follows.

The coagulation time was determined by a modification of the capillary tube method in which the tubes were held suspended in a flask containing water at 39° C. For the differential leucocyte count films were stained by

Pappenheim's method as modified by Piney (1931) and counted by the four-field meander method. For the erythrocyte count blood was diluted to 1:200 in a Thoma pipette. The average difference when counts were checked by another operator was 6 per cent. Following the erythrocyte count the unstained platelets were counted in the haemocytometer. Although not accurate this method was considered to be capable of demonstrating gross changes had these occurred. The fragility of the erythrocytes was determined by adding one drop of blood to tubes containing different concentrations of sodium chloride. The concentration in which complete haemolysis occurred overnight was regarded as the fragility figure. The specific gravity was ascertained by adding a drop of blood to tubes containing different proportions of benzol and chloroform. The specific gravity of the fluid in the tube in which the corpuscles remained suspended was recorded. The corpuscular volume was obtained by filling two small haematocrit tubes with blood, centrifuging these for one hour at 4000 *r.p.m.* and taking the mean of the readings. The sedimentation rate was recorded as the fall in centimetres occurring in 24 hours in a column of blood contained in glass tubes about 4 mm. in diameter.

EXPERIMENTAL

In determining the approximate lethal dose of the toxins for sheep the work of Mason (1935) was very helpful. He determined the lethal dose of the toxins of some anaerobes for mice and sheep and expressed the result as a ratio between the M.L.D. for the mouse and the number of such doses per kg. body-weight which proved lethal for sheep. In our experiments sheep were inoculated in the first instance with a number of mouse lethal doses per kg. which was unlikely to prove fatal. Thereafter others were given a larger dose until a lethal dose was obtained. Altogether 11 sheep were inoculated intravenously with type A toxin, 6 with type C and 9 with type D. The results are recorded in table II.

If the smallest lethal dose of the various toxins for sheep is expressed as the number of mouse lethal doses per kg. body-weight, the ratios mouse M.L.D. : sheep M.L.D. are, for type A, 1:11, for type C, 1:45 and for type D, 1:25. The ratio for type D toxin given by Mason is 1:12.5 but the difference in materials, weight of mice and number and breed of sheep may account for the discrepancy. In all the animals which died the period of illness was short and it was found impossible to produce a chronic clinical condition by administering a sublethal dose of toxin. The result also indicates that sheep are relatively more susceptible than mice to type A toxin.

Before inoculation the sheep were weighed, the wool over the jugular vein on each side of the neck was removed and normal blood samples were obtained. The toxins were injected into the jugular vein and thereafter blood samples were withdrawn from this vessel, 40 c.c. at each bleeding. About 2.0 c.c. were transferred immediately to a small Petri plate for a coagulation test and the preparation of films. The remainder was divided between two bottles. In one the blood was allowed to clot; the other

TABLE II

Toxicity of different types of Cl welchii toxin for sheep

Type of toxin	Sleep no	Breed	Weight (kg)	Dose (mg)	Dose expressed as no of mouse M L D per kg body weight	Result
A	2	S	27.2	100.0	6.0	Lived
	325	S	22.7	81.6	6.0	,
	1000	B F	19.5	120.0	10.0	"
	1	B F	21.3	140.0	10.0	Died, 31 hrs
	59	B F	22.2	146.0	11.0	, 18 hrs 10 mins
	60	B F	21.3	166.0	13.0	, 3 hrs 5 mins
	324	S	33.6	262.0	13.0	, 6 hrs 30 mins
	3	B F	18.1	160.0	14.0	, 9 hrs
	61	B F	21.3	192.0	15.0	, 3 hrs 50 mins
	52	B F	17.7	160.0	15.0	, 2 hrs 5 mins
	944	B f	15.4	200.0	21.0	, 2 hrs 30 mins
C	322	S	59.5	46.0	40.0	Lived
	321	S	63.6	56.2	45.0	Died, 45 mins
	659	H B	48.6	42.0	45.0	, 1 hr 20 mins
	660	H B	65.9	64.0	50.0	, 40 mins
	661	H B	55.4	54.0	50.0	, 4 hrs 5 mins
	636	H B	60.0	72.0	60.0	, 1 hr
D	637	H B	65.4	2.6	10.0	Lived
	629	H B	62.7	3.0	12.5	,
	657	H B	61.3	4.8	20.0	,
	339	B F	20.9	1.9	23.0	,
	658	H B	67.3	6.2	25.0	Died 1 hr 10 mins
	338	B F	25.4	2.4	25.0	, 2 hrs
	639	H B	64.5	7.6	30.0	, 1 hr 45 mins
	650	H B	76.8	12.2	40.0	, 1 hr 30 mins
	634	H B	75.0	12.0	40.0	, 1 hr 15 mins

S = Suffolk

B F = Blackface

H B = Half bred

contained potassium oxalate and sodium fluoride and was shaken vigorously to prevent clotting. This provided sufficient material for the biochemical and physical examination of the blood. Typical examples of the changes following the injection of each type of toxin have been selected for graphic representation in the text*. Post mortem examination of animals which died included the histological examination of the liver, kidneys, lungs and heart wall.

Type A toxin

Symptoms

Sheep inoculated with a lethal dose of type A toxin developed an increased respiratory rate shortly after injection. This became progressively more marked until there developed an acute dyspnoea accompanied by the appearance of blood stained froth at the nostrils. There was incoordination of gait and muscular weakness, which increased until the animal was unable to stand.

* The detailed records of all the observations made on the blood obtained from the sheep under experiment have been deposited in the General Library, British Museum (Natural History), London, S W 7

Fluid faeces were passed, tinged with blood in no. 59, and marked hæmoglobinuria developed in all cases. No significant variation in body temperature occurred except in no. 324, whose temperature rose from 102.6° F. at the time of inoculation to 108° F. fifteen minutes before death. Death occurred suddenly in all cases without evidence of coma.

Post-mortem findings

A characteristic feature was the presence of hæmorrhages in different parts of the body. In the alimentary tract the mucous membrane of the abomasum almost invariably showed hæmorrhages ranging from petechiæ to extensive extravasations. Petechial hæmorrhages were noticed on both surfaces of the small intestine and in no. 59 the contents were chocolate coloured. The liver and kidneys were much darker than normal. The urine was dark, due to blood pigment. The mucous membrane of the bladder appeared normal. The condition of the lungs ranged from acute congestion to hæmorrhagic œdema. In several cases the trachea and bronchi were filled with blood-stained froth. Hæmorrhages under serous membranes were usually present, particularly over the diaphragm, mesentery and omentum. Subcutaneous sub-endocardial and sub-epicardial hæmorrhages were present in all cases.

Histologically the liver showed necrobiotic changes together with round-celled infiltration of Glisson's capsule. Changes were most apparent round the central vein. The lungs showed some hyperæmia of the alveolar capillaries with œdematous exudation into the alveoli in some areas. In one section a small hæmorrhage was observed. The kidneys showed marked nephrosis. A large amount of colloidal material was seen in the form of hæmoglobin cylinders within the tubules and as an extravasation between the tubules. This material was most apparent in the lumen of the convoluted tubules in the subcapsular part of the cortex but was also observed in Henle's loop and in some collecting tubules. In the heart sub-endocardial hæmorrhages were present, erythrocytes lying free under the endocardium and permeating between the muscle fibres and around Purkinje's fibres to a depth of 1.2 mm. The mucous membrane of the abomasum in most cases was congested and sections prepared from the most acute lesions revealed intense congestion of the capillaries and larger blood vessels, with extravasation of blood into the mucous membrane.

Physical changes in the blood

The changes which occurred in the blood of sheep no. 61 after inoculation are typical and are shown graphically in fig. 1.

It will be seen that the number of red corpuscles fell from

9 million to 2 million per c mm in 3 hours. This was accompanied by a fall in specific gravity from 1055 to 1035, an increase in sedimentation rate, a fall in the total cell volume from 30 per cent to 4 per cent and an increase in the fragility of the red cells. No constant change in the clotting time was observed, although sheep

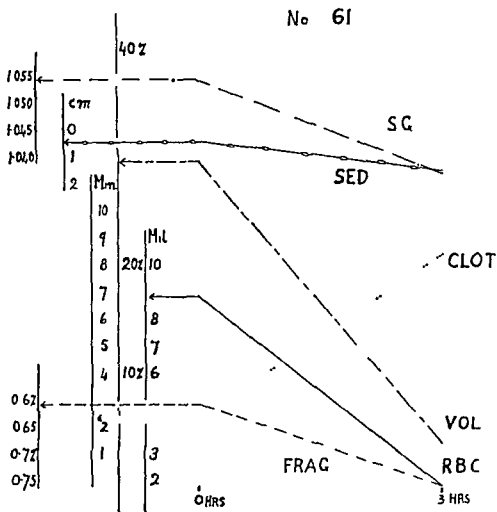


FIG 1—Physical changes in the blood following the injection of type A toxin

no 61 showed an increase. A rise in the colour index occurred in all sheep which received a lethal dose. In films a few cells were seen showing polychromasia and there was an appearance of anisocytosis but no measurements were made.

Leucocytic reaction

Fig 2 depicts the leucocytic changes in sheep which received a lethal dose. Agranulocytosis developed within an hour of inoculation (no 324). This was followed within a few hours by a progressive increase in granulocytes until, as in no 59, 11 hours after injection the neutrophils had returned to about half their pre inoculation figure and by 18 hours a further increase was recorded. The polynuclear index of no 59 before inoculation was 2.65, 11 hours after inoculation it fell to 1.76 and rose again to 1.95 eighteen hours after, which suggests that the return towards the normal count was accompanied by a slight shift to

the left. Little change occurred in the absolute number of lymphocytes, while the monocytes showed a tendency to decrease. In one animal which received a non-lethal dose there was a slight absolute neutrophilia 2 hours after inoculation.

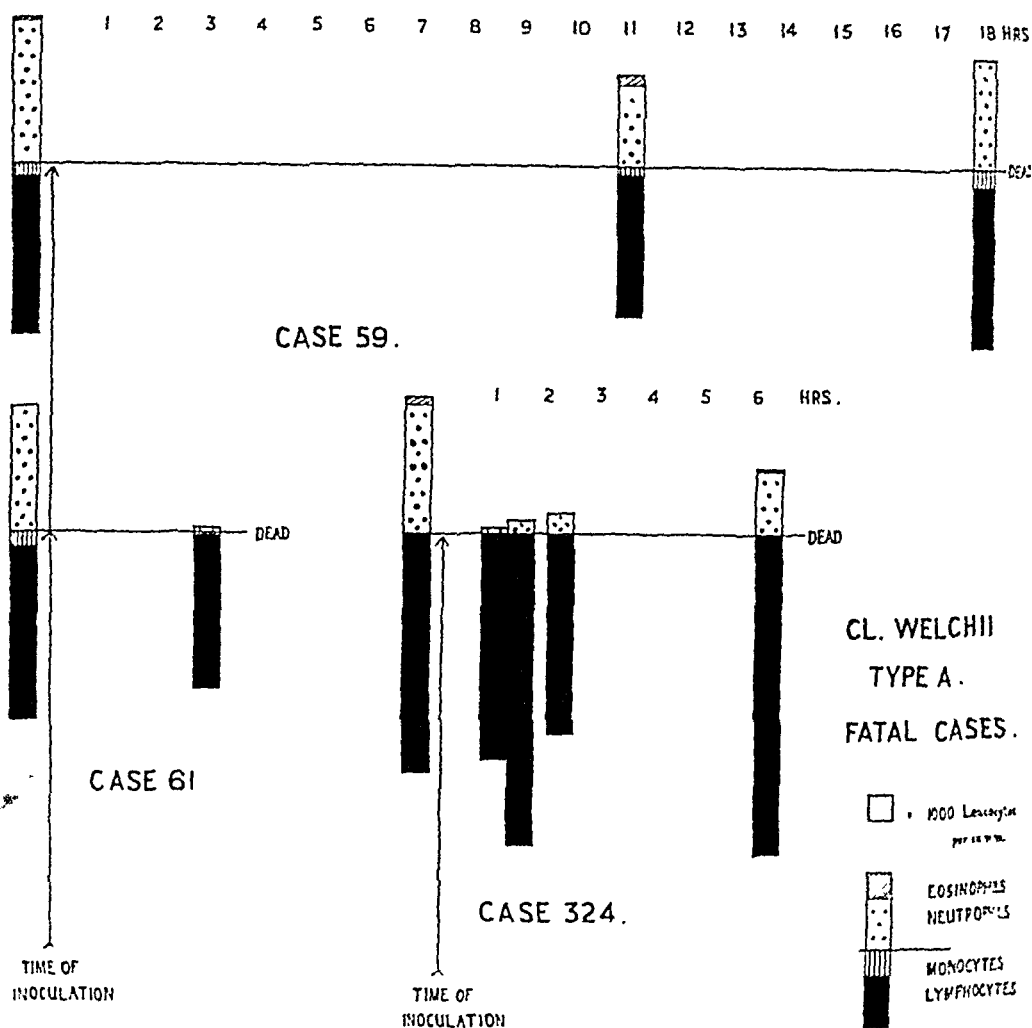


FIG. 2.—Leucocytic reactions in sheep which received a lethal dose of type A toxin.

Biochemical changes in the blood

Fig. 3 depicts the sequence of changes in sheep 61 and shows that the most striking changes were the rapid fall in the hæmoglobin and serum calcium, the hæmoglobin from 10.1 to 4.0 g. per 100 c.c. and the calcium from 8.00 to 5.06 mg. per 100 c.c. These falls occurred whether the animal lived or died. There was no significant change in the other constituents. The sugar

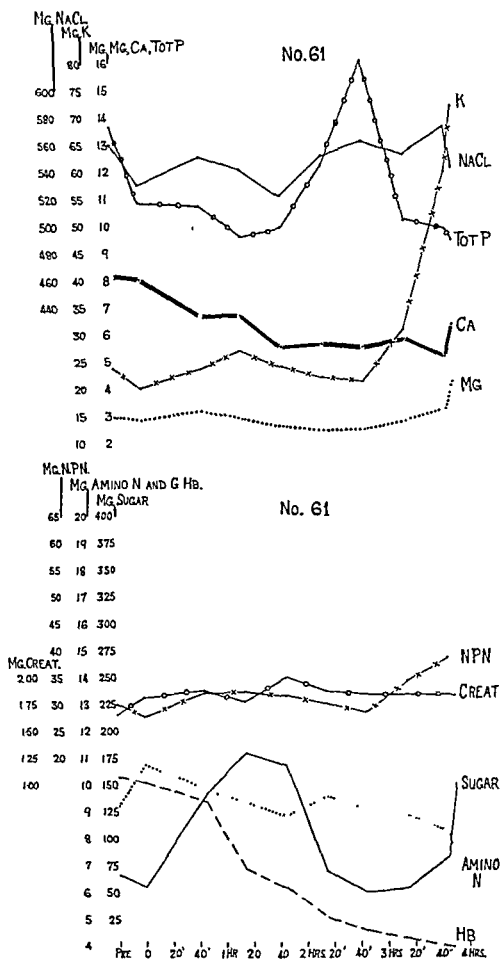


FIG. 3.—Biochemical changes in the blood following the injection of type A toxin.

rose in no. 61 but fell considerably in other cases, *e.g.* no. 324; the creatinine rose considerably in most cases. Magnesium and potassium showed much fluctuation but usually rose just before death. The non-protein nitrogen and amino-nitrogen fluctuated widely but the chlorides remained constant. The phosphates, both inorganic and total, remained constant in most cases, with a slight tendency to rise occasionally.

Type C toxin

Symptoms

Sheep which died showed symptoms of dyspnoea and inco-ordination of gait similar to those injected with type A toxin. The outstanding difference was the absence of hæmoglobinuria.

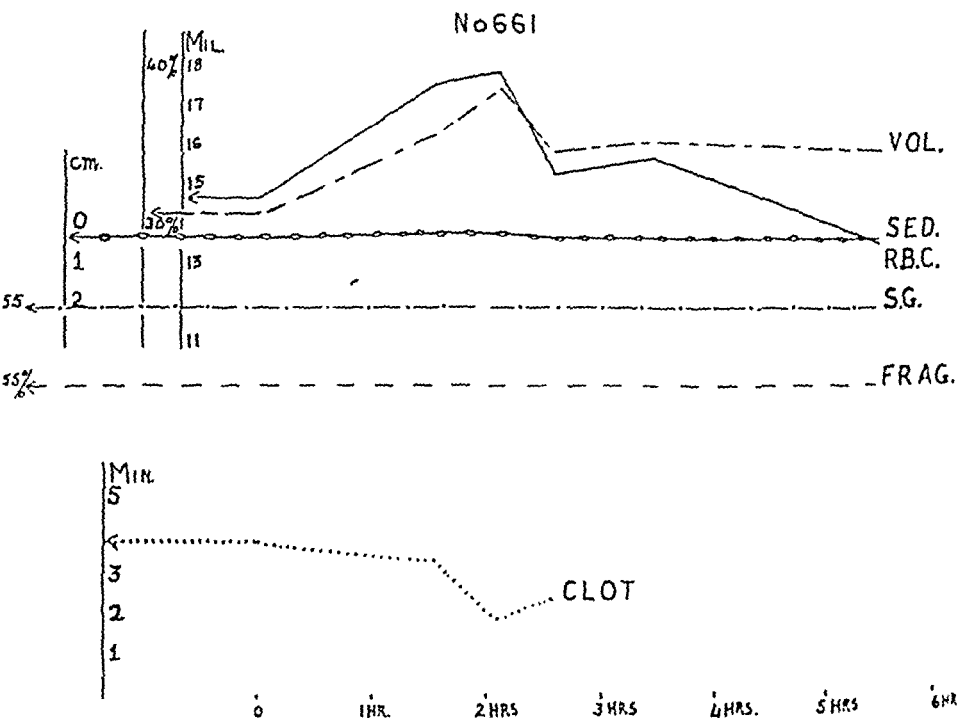


FIG. 4.—Physical changes in the blood following the injection of type C toxin.

Post-mortem findings

The only constant change observed was the presence of petechial hæmorrhages under the endocardium of the left ventricle. In no. 321 the hæmorrhage took the form of an extensive extravasation. In no. 660 there was one large hæmorrhage under the visceral pleura and the serous surface of the abomasum of no. 661 was congested.

Histologically the œdematous condition of the lung, so noticeable with type A toxin, was absent. Some of the alveoli contained erythrocytes, dust cells and plasma: exceptionally one or two around a bronchiole contained œdematous exudate. In the kidney hyaline droplets were observed in Bowman's capsules and in the lumen of the tubules. Slight extravasations of blood were seen between the tubules in the cortical rays and in one case definite hæmorrhages were present among the convoluted tubules.

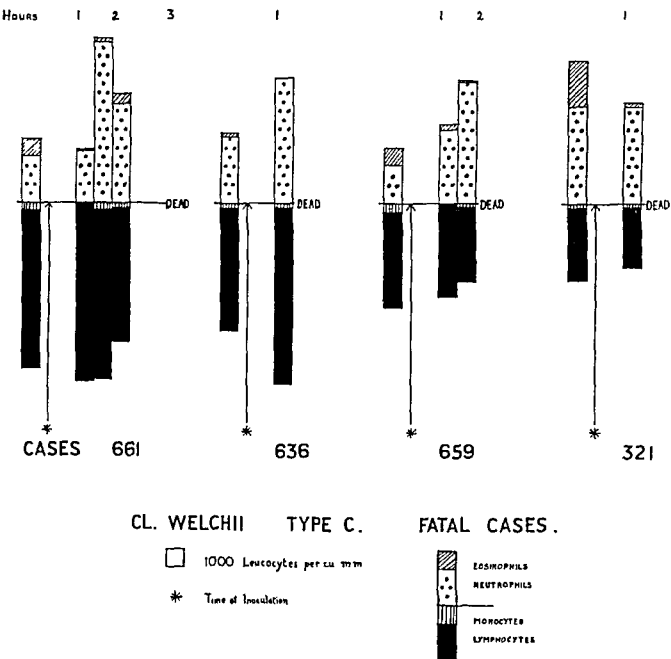


FIG. 5.—Leucocytic reactions in four sheep which received a lethal dose of type C toxin.

Physical changes in the blood

Sheep no. 661 (fig. 4) is representative of the effect of C toxin on the blood. No marked alteration occurred apart from a temporary rise in the number of red blood corpuscles and this was not observed in all the animals. In 3 of the 5 animals that

died the fragility of the erythrocytes was increased, and in three in which the clotting time was observed, this was decreased. No significant change occurred in the colour index and films showed an unaltered appearance of the erythrocytes and platelets.

Leucocytic reaction

Although a slight fall in the number of granulocytes occurred in nos. 661 and 321, the agranulocytosis which characterised the reaction to type A toxin was not apparent even one hour after inoculation (fig. 5). The slight reduction in granular cells in no. 661 was followed by an absolute neutrophilia, and, except in no. 321, the final reaction took the form of a leucocytosis in contrast to the leucopenia which followed the injection of type D toxin. The absolute number of lymphocytes was little altered, two cases showing a slight increase and the other two a slight decrease. The monocytes tended to diminish in number. No. 322, which received a non-lethal dose, had a very high leucocyte count before inoculation, and hence the reaction two hours later appears only as a relative and not an absolute neutrophilia.

Biochemical changes in the blood

No characteristic picture was obtained. The data from a typical case, no. 661, are shown in fig. 6. The majority of the blood constituents remained constant—the chlorides, inorganic and total phosphorus, potassium, calcium and magnesium. Slight fluctuations only took place. In some cases the sugar rose gradually till death, e.g. no. 659, but in no. 661, as in others, it rose immediately after injection and fell again suddenly to reach a normal value before death. In one or two cases it remained constant. The non-protein nitrogen and amino-nitrogen showed small increases in some animals but in others remained practically constant. The creatinine remained constant in most cases but in nos. 661 and 659 a slight increase took place. The hæmoglobin remained constant in all sheep in which death occurred within a few hours, but in no. 3, which survived, it fell gradually within 48 hours from 10·8 to 7·85 g. per 100 c.c., and even two months after injection was found to be only 6·65 g. per 100 c.c.

Type D toxin

Symptoms

Dyspnœa and incoordination of gait were again the prominent symptoms. In addition, there were symptoms suggestive of disturbance of nerve function such as the twisting of the head to one side and pleurothotonos. Tremors were observed along the

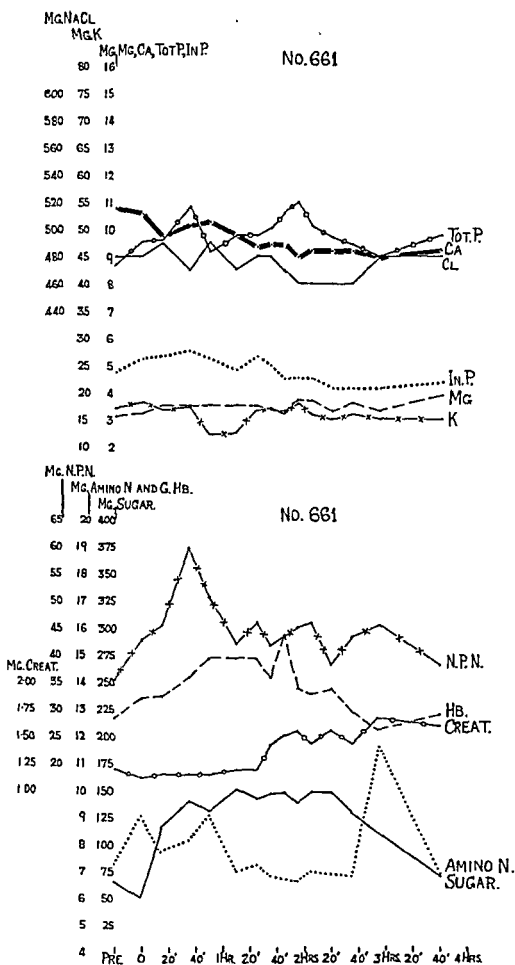


FIG. 6.—Biochemical changes in the blood following the injection of type C toxin.

muscles of the back and the animals showed a desire to make use of fixed objects as aids in support. Death often occurred after a short period of coma.

Post-mortem findings

Again the most constant lesions were sub-endocardial and sub-epicardial hæmorrhages, mainly along the course of the coronary vessels. The lungs appeared congested and the blood vessels prominent. The liver was darker than normal owing to venous congestion.

Histologically, in addition to the usual liver damage observed after injection of the other two types of toxin, the sinusoids of the liver were obviously congested. This change was sufficiently marked to distinguish these liver preparations from those obtained from sheep which had died from the other toxins. The changes in the lung and kidney were similar to those due to type C toxin.

Physical changes in the blood

These were indicative of an anhydræmia. Fig. 7 depicts the findings in sheep no. 338, which is representative of the group.

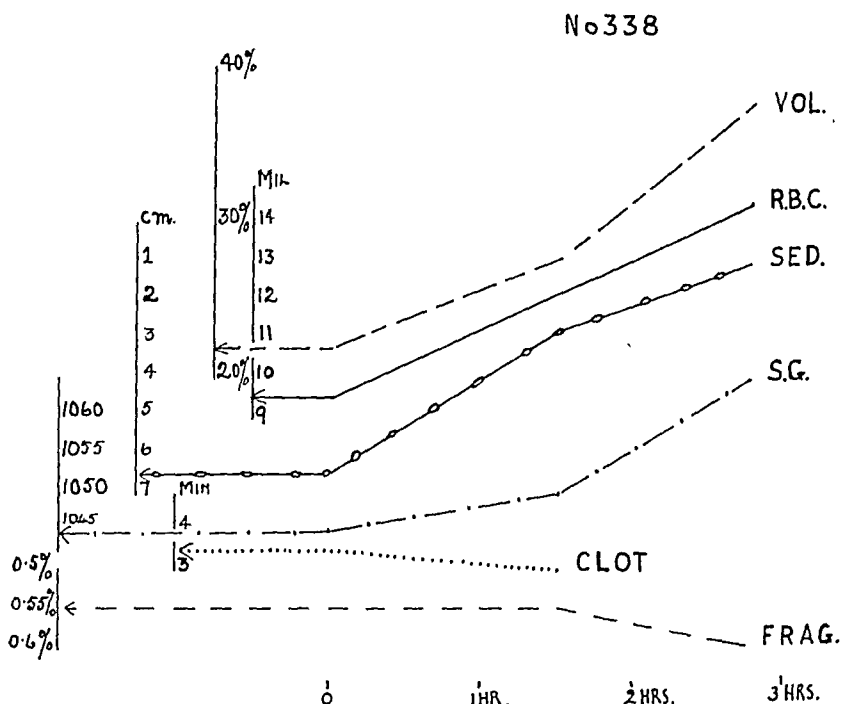


FIG. 7.—Physical changes in the blood following the injection of type D toxin.

A definite polycythæmia occurred, the red cell count rising from a pre-inoculation figure of 9.5 million to 14.6 million per c.mm.

This was accompanied by a rise in specific gravity from 1045 to 1065 and an increase in the total cell volume from 22 to 38 per cent. There was a decrease in the sedimentation rate and the blood clotted more rapidly. In two of the five sheep which died the fragility of the red cells was slightly increased. No significant change occurred in the colour index. In the four animals which received a sub lethal dose there was a slight oligocythæmia, and the specific gravity and total cell volume decreased proportionally. Films showed no abnormality of the erythrocytes or platelets except in one case, which showed anisocytosis and a single polychromatic cell.

Leucocytic reaction

Fig 8 shows that in all four animals which received a lethal dose there was a fall in the number of granular cells but much

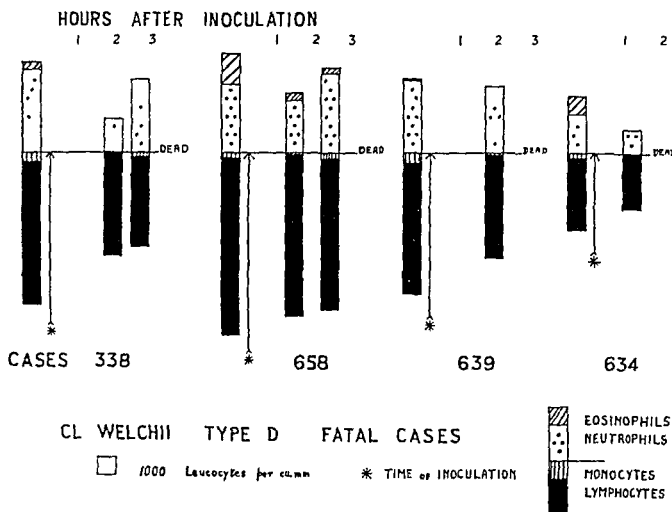


FIG 8 —Leucocytic reactions in four sheep which received a lethal dose of type D toxin

less than with type A toxin. As with type A, the reaction was greatest one hour after inoculation and tended to decrease after 2-3 hours. This reaction was in distinct contrast to the neutrophilia after type C toxin. Each of the four animals receiving a lethal dose showed a decrease in the absolute number of lymphocytes,

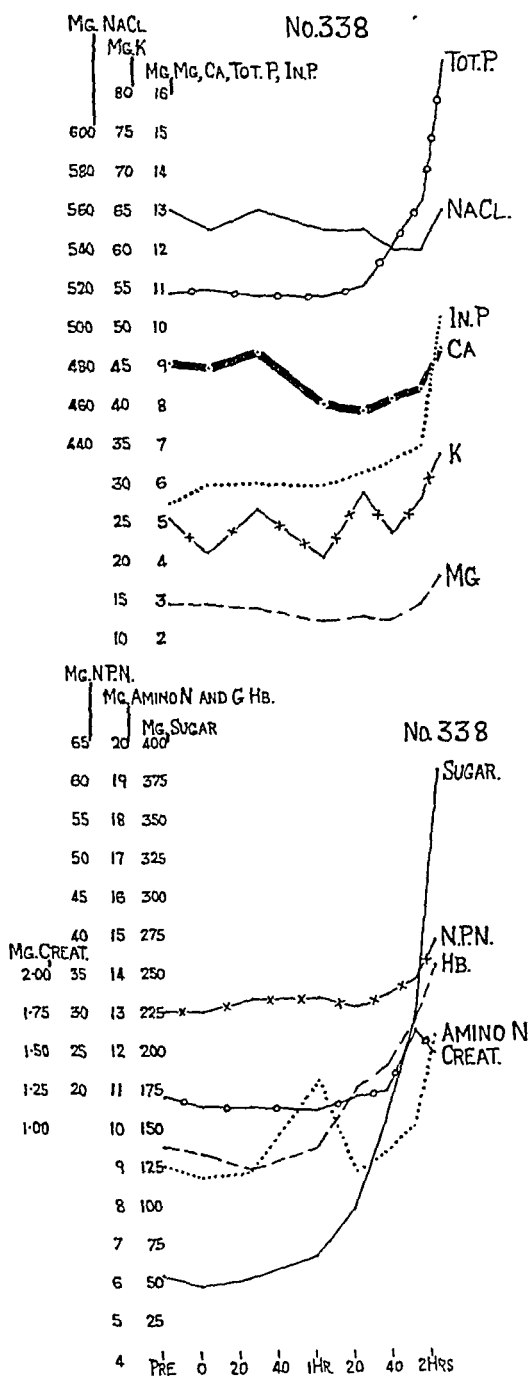


FIG. 9.—Biochemical changes in the blood following the injection of type D toxin.

and in nos 338 and 658, from which three samples were obtained, this change was progressive. Since a decrease in the monocytes also occurred the sum of the reactions represented a leucopenia.

As with the other toxins, sheep which received a non-lethal dose showed an absolute neutrophilia two or more hours after inoculation.

Biochemical changes in the blood

Type D toxin produced the greatest effect on the blood constituents but only if a lethal dose were injected. The data from sheep no 338 are shown in fig 9. Most of the blood constituents showed a large increase after inoculation. The rise was most marked in the hæmoglobin and sugar. In no 658 the hæmoglobin rose from 13.0 to 19.0 g and the sugar from 55.5 to 121.2 mg per 100 c.c., while in no 338 (fig 9) the hæmoglobin rose from 9.3 to 14.28 g and the sugar from 47.1 to 380.1 mg per 100 c.c. The phosphate, both inorganic and total, magnesium, potassium, non-protein nitrogen, amino acid nitrogen and creatinine all showed substantial rises. The calcium either remained constant or fell slightly. The chloride usually remained constant but in some cases fell to a very slight extent, as in no 658. The picture described above and represented in fig 9 is that of a typical anhydræmia in which the organic constituents of the blood are usually considerably increased while the inorganic constituents are either slightly increased or even slightly decreased, the chloride usually falling to a significant extent. With type D toxin the blood picture described was found in all sheep which died. In those which were given a sublethal dose both the inorganic and total phosphorus tended to fall slightly, while the other constituents remained constant or fluctuated to a small degree. The hæmoglobin content of no 339, which survived, fell rapidly to 6.83 g after 48 hours and even one month after inoculation had not regained its normal value.

SUMMARY

The symptoms, intra-vitam effects on the blood and post-mortem findings following the intravenous inoculation of sheep with the toxins of *Cl. welchii* types A, C and D have been compared. With the exception of hæmoglobinuria, which occurred only in sheep inoculated with type A, no marked difference in symptoms was observed. Type A toxin produced a rapid fall in the number of red blood corpuscles with a corresponding fall in hæmoglobin, specific gravity and total cell volume, an increase in the sedimentation rate and an increase in red cell fragility. No specific blood changes followed the inoculation of type C toxin. Type D toxin produced a rise in the number of red corpuscles with a corresponding

rise in hæmoglobin, specific gravity and total cell volume. There was a decrease in sedimentation rate and, with the exception of calcium and chloride, a marked rise in the inorganic and organic constituents of the blood. Anisocytosis and slight polychromasia followed the injection of type A toxin but was not observed after a lethal dose of either C or D. Wallbach (1935) reviews work which showed that the injection of toxins was followed by a transient agranulocytosis and this in turn by a neutrophilia. In the case of the *welchii* toxins A and D there was a temporary fall in the number of granular cells followed by a return more or less to the original figure. Two hours after inoculation agranulocytosis was still present with type A toxin, while with type D the granular leucocytes were still slightly lowered; with type C there was a slight neutrophilia. Two hours after inoculation with non-lethal doses of all three types there was a neutrophilia. Type D toxin was exceptional in that it also caused a reduction in the number of lymphocytes. The out-standing histological differences were that sheep killed with type A toxin showed an œdematous exudate in the alveoli of the lungs and a large amount of colloidal material in the kidneys in the form of hæmoglobin cylinders within the tubules and extravasated between the tubules, whereas these changes were not observed in sheep inoculated with types C and D. Sheep inoculated with type D showed marked congestion of the sinusoids in the liver: this was absent in sheep which had received types A and C.

CONCLUSION

As a result of these experiments it may be concluded that whereas the toxin of *Cl. welchii* type C produces no specific blood changes, that of type A produces a marked hæmolytic anæmia, while that of type D produces an anhydræmia.

We are indebted to Dr R. A. O'Brien for sera and to Professor T. Dalling and Dr R. F. Montgomerie for kindly placing cultures at our disposal.

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A SURVEY OF THE TYPES OF *CL. WELCHII* PRESENT IN SOIL AND IN THE INTESTINAL CONTENTS OF ANIMALS AND MAN

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THE detection of the toxin of *Cl. welchii* type D in, and the isolation of the associated organism from, the intestinal contents of a few acute cases of grass sickness in horses has already been reported (Gordon, 1934). In an endeavour to assess the significance of that finding it was considered desirable to make a survey of the types of *Cl. welchii* normally present in farm soil and in the intestinal contents of man and several animal species. In addition, 174 filtrates prepared from intestinal contents were examined for the presence of toxin. The work involved is the subject of this paper, which records the isolation and typing of 1343 cultures of *Cl. welchii*.

Within recent years the typing of the *Cl. welchii* group of spore-bearing anaerobes and the role of the different types in animal disease have been the subject of considerable study by various workers in different parts of the world. This work has been reviewed by a number of authors and most recently by McCoy and McClung (1938) and Dalling and Ross (1938). Although types A, B, C and D have been incriminated in the causation of disease in animals there are few records of the isolation of types other than A either from soil or from the intestinal contents of normal animals and man. Dalling (1928) isolated type B from 4 out of 6 samples of soil obtained from farms on which lamb dysentery was prevalent and Bennetts (1932) isolated type D from the soil of paddocks in which sheep had died of entero-toxæmia, while he failed to detect it in soils on which this disease was absent. Borthwick (1937) found only type D in the intestinal contents of each of three guinea-pigs, both types A and D in similar material from six dogs and only type A in the intestinal contents of two rabbits and six human subjects. Borthwick and Gray (1937) also record that five cultures of *Cl. welchii* isolated from the faeces of patients suffering from pernicious anæmia were type A.

Origin of material examined

Soil. The samples of soil examined were collected in the counties of Angus and Aberdeen from fields on which horses had developed acute grass sickness, with the exception of one from a field adjoining this laboratory. A small portion taken from about $\frac{1}{4}$ inch below the surface was transferred to a sterile test-tube by

means of a sterile scalpel. Areas of the field obviously contaminated by animal faeces were avoided.

Intestinal contents. This material was obtained from a number of different animal species and from human subjects. It comprised a mixture of the contents of the large and small intestine collected in a sterile container. The material of animal origin was taken in some cases from apparently normal animals which had been destroyed and in others from animals which had died from diseases not associated with *Cl. welchii* infection. The material of human origin was collected from subjects in the post-mortem room of a general hospital.

Bacteriological methods

Isolation of Cl. welchii from soil. The samples were stored at room temperature for several months and were dry when used. About 0.5 g. of dry soil was placed in a tube of cooked meat (horse flesh) medium containing 1 per cent. Bacto-peptone and one-half to one-third of its volume of minced horse flesh. The pH was 7.6 to 7.8. The tube was incubated at 37° C. until gas production became obvious, usually in about 4-6 hours. Blood agar plates (sheep blood) were inoculated from this young culture and incubated anaerobically in a McIntosh and Fildes jar overnight. Hæmolytic *welchii*-like colonies were transferred to tubes of cooked meat medium and incubated overnight. Cultures resembling *Cl. welchii* were transferred to cooked meat medium and litmus milk and again incubated overnight. Those which produced a "stormy clot" in litmus milk were provisionally accepted as *Cl. welchii* and the culture fluid from the corresponding cooked meat tube was used for identifying the type of toxin present.

Isolation of Cl. welchii from intestinal contents of animals. The same technique was employed as for soil samples, approximately 0.5 c.c. of mixed bowel contents being used as the primary inoculum.

Isolation of Cl. welchii from intestinal contents of man. It early became obvious that *Cl. welchii* was more difficult to isolate from the intestinal contents of man than from those of animals. Accordingly the technique was altered to take advantage of the fact that *Cl. welchii* sporulates in a sugar-free medium. The bowel contents were inoculated into a tube of alkaline egg medium, incubated anaerobically overnight and then heated at 80° C. for 30 minutes. Subcultures were made into cooked meat, transfers made to blood agar plates and the isolation continued as before.

Antitoxins used

The antitoxins employed for typing cultures were prepared in horses. Three were kindly supplied by the Wellcome Physiological Research Laboratories. They were labelled type A, G.G. 3269; type C, R. 3589; type D, R. 2466. The remainder were prepared in horses at this Institute and were as follows.

Type A, N.C.T.C. 26. Prepared from a type A culture, N.C.T.C. 26, received from Professor Dalling in 1935.

Type B, A.D.R.A. Prepared from several strains of *Cl. welchii* recently isolated from cases of lamb dysentery. This serum was capable of neutralising the toxin of type B in all phases of its growth.

Type D, H. 35/4. Prepared from a strain of *Cl. welchii* type D isolated in 1935 from the bowel contents of a horse affected with acute grass sickness.

Typing of cultures

The various strains isolated were typed according to the classification of Wilsdon (1931-1932-33), who recognised four distinct serological types designated A, B, C and D. Antigenically type A is the simplest, since its antitoxin will neutralise only type A toxin while type B is the most complex, since its antitoxin will neutralise the toxin of all four types. The antitoxin of type C neutralises both A and C toxins and that of type D both A and D toxins.

Soil cultures Because of the association of type D with grass sickness in horses the technique adopted in the examination of soil cultures was devised for the detection of type D. Accordingly the cultures were incubated for 4 days (the time required for maximum development of type D toxin) then centrifuged at 3000 *rpm* for 30 minutes, after which 1.0 c.c. of supernatant fluid was placed in each of two tubes. To one of the tubes 0.2 c.c. of type A antitoxin was added and to the other 0.2 c.c. of type D antitoxin. After contact for 30 minutes three mice were inoculated intravenously, one with 0.6 c.c. of the type A mixture, one with the same dose of the type D mixture and a control with 0.5 c.c. of the culture fluid alone. If after 4 days all the mice died or if the mouse which received type D antitoxin survived while the other two died further confirmatory tests were made with antitoxins of types A, B, C and D, using a larger number of mice. If the control mouse died and both mice which received antitoxin survived, the culture was accepted as type A, while if all the mice survived, accurate typing was not proceeded with.

Cultures from intestinal contents The cultures from intestinal contents were incubated overnight in meat broth and tested in the same manner as the soil cultures, except that the antitoxins used in the preliminary tests were types A and B instead of types A and D. If both the mice which received antitoxin survived for 2 days after inoculation, while the control died within the same period, the culture was accepted as type A. If the control mouse survived, the culture was subcultivated twice daily for several days, after which sufficient toxin was usually produced to enable the typing to be completed. If after these tests the only mouse which survived was the one which received type B antitoxin, further tests on larger groups of mice were made with antitoxins of types A, B, C and D until the culture was finally assigned to a definite type.

The types of Cl welchii isolated from soil

Altogether 196 cultures from 43 samples of soil were examined, with the result shown in table I.

TABLE I

Analysis of the typing of cultures of Cl welchii from soil

Year	No. of soil samples	Total no. of cultures	No. of type A or non toxic cultures	No. of type D cultures
1936	20	87	80	7
1937	23	109	109	0
Total	43	196	189	7

Although 7 type D cultures were isolated in 1936 from 4 different fields not one was isolated in 1937 despite the increased number of cultures examined. Of the 4 samples from which type D was isolated, 3 were obtained from fields on which horses had developed acute grass sickness. The fourth was taken from a field adjoining the laboratory in which at least two cases of entero-toxæmia in adult sheep were known to have occurred.

*Types of Cl. welchii isolated from the intestinal contents
of man and animals*

Table II summarises the result of typing 1147 cultures from the bowel contents of several species of animals and man. It will be observed that, with the exception of three type B and ten type D cultures, all were of type A. The bovine from which the two type D cultures were isolated was a normal animal killed for human consumption. The sheep which yielded one type D culture was killed while affected with louping-ill. The rabbit which yielded seven type D cultures belonged to the normal stock at the

TABLE II

*Types of Cl. welchii isolated from the intestinal contents of
animals and man*

Species	Number examined	Number yielding <i>Cl. welchii</i>	Total cultures examined	Type A	Type B	Type D
Cattle . .	25	23	160	158	0	2*
Sheep . .	23	23	170	169	0	1
Pigs . .	28	26	191	191	0	0
Dogs . .	21	17	110	110	0	0
Cats . .	7	7	48	48	0	0
Rabbits . .	15	13	105	95	3*	7*
Guinea-pigs . .	17	16	114	114	0	0
Poultry . .	13	12	88	88	0	0
Man . .	25	25	161	161	0	0
Total . .	174	162	1147	1134	3	10

* Isolated from one animal in each case

Institute. This animal died suddenly and, although no toxin was detected in a filtrate prepared from the bowel contents, the post-mortem appearances suggested that death was due to entero-toxæmia. In a second stock rabbit which died suddenly three weeks after the first, type D toxin was detected in a filtrate prepared from the bowel contents, but 15 cultures isolated from the bowel contents were all of type A. The rabbit from which the three type B cultures were isolated was a wild rabbit killed in its warren by carbon monoxide inhalation.

The identity of the type D cultures was established by the fact that the culture fluid could be neutralised only by type D antitoxin. In the type B cultures the toxin formed was tested both after 16 hours' and after 4 days' growth. At 16 hours the culture fluid was neutralised by both types B and C antitoxin, while after 4 days' growth neutralisation was effected only by type B antitoxin. These results were confirmed on several occasions.

*Tests for the presence of toxins in filtrates
of intestinal contents*

All the samples of intestinal contents examined bacteriologically were also tested for the presence of toxins by intravenous inoculation of mice with filtrates through paper pulp. In some cases the bowel contents were suitable for filtration without dilution, in others the material required to be mixed with an equal volume of saline. The filtrates were injected intravenously in doses of 0.75, 0.5 and 0.1 c.c. Of 174 examined only one, obtained from the second stock rabbit referred to above (p. 274), was lethal for mice in a dose of 0.5 c.c. The toxin present was determined as type D, being neutralised only by type D antitoxins. Twelve other filtrates killed mice in a dose of 0.75 c.c., but because of the large volume of this lethal dose it was not possible to determine by neutralisation tests whether or not the toxic substance was *welchii* toxin.

*Tests for the presence of hæmolysin in culture filtrates
of the different types of Cl. welchii isolated*

It is generally recognised that type A toxin is actively hæmolytic *in vitro* when added to a suspension of red corpuscles in saline,

TABLE III
Hæmolysin production by different types of Cl. welchii

Type	Culture	M H D	Culture	M H D	Culture	M H D
A	Bovine XIV. 3	0 005	Bovine XVI. 2	0 005	Sheep V. 8	0 005
	Sheep VII. 1	0 025	Pig IV. 4	0 005	Pig X. 2	0 0075
	Dog III. 1	0 025	Dog V. 3	0 01	Cat I. 4	0 025
	Cat II. 2	0 025	Rabbit III. 3	0 0075	Rabbit VII. 7	0 01
	G. pig X. 5	0 0075	G. pig XV. 4	0 01	Fowl VII. 2	0 005
	Fowl IX. 2	0 0075	Human XXII. 2	0 0075	Human LVI. 6	0.01
B	Rabbit XV. 1	>0 5	Rabbit XV. 2	>0 5	Rabbit XV. 3	>0 5
D	Bovine XXI. 1	..	Bovine XXI. 2	..	Sheep X. 3	0 25
	Rabbit II. 2	>0 5	Rabbit II. 3	0 5	Rabbit II. 4	>0 5
	Rabbit II. 5	>0 5	Rabbit II. 6	>0 5	Rabbit II. 7	>0 5
	Rabbit II. 8	>0 5	Soil M L. 2	0 1	Soil M L. 3	0 1
	Soil S C. 2	0.25	Soil A D.R.A. 1	0.25	Soil S S P. 3	0 075
	Soil S S P. 1	0 075	Soil S S P. 6	0 075

M H D. = amount of culture fluid (c.c.) required to produce 50 per cent. hæmolysis in a 6 per cent. suspension of sheep red blood cells.

while types B and D are less active. Two type A cultures from each species and most of the cultures of the other types isolated were tested for their hæmolytic power, using a method similar to that described by Henry (1922). A 6 per cent. suspension of sheep red corpuscles in saline was employed. After addition of the culture fluid the tubes were incubated for one hour in a water-bath at 37° C., stored overnight in the cold and a reading taken the following morning. The result is shown in table III, from which it will be observed that the type A cultures were more hæmolytic than those of types B and D.

Discussion

The isolation of *Cl. welchii* type D from the soil of certain fields suggests that this organism may sometimes gain access to the digestive tract of herbivorous animals grazing on such pastures. That this does occur occasionally is exemplified by the isolation of type D from the intestinal contents of a normal bovine and of a sheep suffering from louping-ill. The presence of this organism in the intestinal contents of an apparently normal bovine is worthy of note, since it suggests that entero-toxæmia might well be the cause of death in certain unexplained fatal illnesses of short duration in cattle.

The detection of type D toxin in the bowel contents of a rabbit which died suddenly and the isolation of the associated organism from another rabbit which died under similar circumstances indicate that entero-toxæmia may be a cause of acute illness, with rapidly fatal outcome, in these animals. That the apparently healthy intestine of a wild rabbit should harbour *Cl. welchii* type B is of importance in relation to the epizootiological aspect of lamb dysentery, since it raises the possibility that the wild rabbit may be a factor in the dissemination of this disease.

Summary

1. Of 196 strains of *Cl. welchii* isolated from 43 samples of farm soil, 7 were of type D and the remainder of type A.
2. Of 1147 strains isolated from the intestinal contents of man and of several species of animals, 1134 were of type A, 3 of type B and 10 of type D. The type B strains were isolated from a wild rabbit; of the D strains one was isolated from a sheep, two from a bovine and seven from a domesticated rabbit.
3. Of 174 filtrates prepared from intestinal contents, the toxin of *Cl. welchii* was detected in only one, a sample from a rabbit which died after an acute illness. This was of type D and the animal probably died of entero-toxæmia.

We are indebted to Dr W G Miller of the Royal Infirmary, Edinburgh for the material of human origin, to Mr E J Sewell of the City Veterinary Department, Edinburgh and Mr W M Henderson and Mr J L Wilson of the Royal (Dick) Veterinary College, Edinburgh, for some of the material of animal origin, to Dr R A O'Brien, Director of the Wellcome Physiological Research Laboratories for supplies of antitoxins and to the Director of this Institute, Dr J Russell Greig, for the interest he has taken in this work.

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OBSERVATIONS ON LIVING VACCINIA VIRUS IN THE CORNEAL CELLS OF THE RABBIT

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(PLATES XXIX AND XXX)

As long ago as 1892 Guarneri inclusion bodies were demonstrated in stained sections of rabbit cornea (Guarneri, 1894) and Paschen described elementary bodies in 1906. Ewing (1904-05) showed that Guarneri bodies contained granules, and Volpino (1907) described mobile granules of high refractivity within corneal cells. These structures appeared within 24 hours of inoculation and seemed to bear a direct relationship to the degree of infection. The development of the infection has been studied extensively by means of numerous staining methods, in the cornea Boing (1920) stained acidophilic granules inside the Guarneri bodies and Schutz (1925-26) described the discharge of a great number of granules from disintegrating Guarneri bodies. Nauck and Robinow (1935-36), Haagen and Kodama (1937), Rhodes and van Rooyen (1937a) and Bland and Robinow (1939) studied the behaviour of the virus in tissue cultures and suggested an evolutionary cycle. Further evidence in the same direction from observations of the virus in the chorio-allantoic membrane of the developing chick embryo has been provided by Goodpasture, Woodruff and Buddingh (1932), Herzberg (1936a and b, 1937), Tang and Wei (1937) and Himmelweit (1938).

The observations described in the present article deal mainly with the direct examination of the development of the unstained vaccinia virus in the living corneal cells of the rabbit under dark ground illumination.

Technique

The eye of the rabbit was inoculated in the usual manner by scarification with a strain of vaccinia virus kindly made available by Professor James McIntosh. After the desired interval of time the animal was killed and its eyes fixed in position by clamping their connections with artery forceps.

The next step depended on the degree of development of the lesion. If vaccinal keratitis was well advanced (as a rule after 3 days) thin tangential shavings could readily be cut with a sharp iridectomy knife, but when the lesion was less developed and the cornea still remained tough and resistant, shavings could not be obtained. In such cases a sufficient number of cells could be detached by scraping the surface with the blade of the knife. Control preparations from the uninoculated cornea were of necessity of this type.

The material obtained was placed in a drop of physiological salt solution or better Tyrode solution on a thin slide, covered with a coverslip and gentle pressure applied to expel excess of liquid. The coverslip was then sealed with paraffin wax in the usual way to prevent evaporation. Observations were carried out either at room temperature or on the warm stage at 37° C. and in many cases changes occurring during a period of several days were followed and recorded. An optical system giving a high degree of resolution with dark ground illumination is essential. Details of this equipment have been previously described (Merling-Eisenberg, 1927-28, 1935 *a* and *b*, 1937 *a* and *b*, 1938).

Results

The appearance of the preparation depends essentially on the degree of development of the lesion. About one-fifth of the thickness of the cornea is composed of polygonal epithelial cells, and when the lesion is advanced, the first shaving consists almost entirely of the large flat cells of the surface epithelium. These are easily torn apart and appear in the preparations singly, in small groups or in sheets. In the second and later shavings the majority of the cells obtained are the smaller polygonal cells of the deeper layers of the cornea. In the final or deepest shavings connective tissue elements are included. In scrapings from earlier lesions fewer cells are usually obtained.

The first shavings or scrapings always contain some infected cells if the knife has passed through the inoculated area, since these cells come off more easily than those of completely healthy tissue. If the lesion is too far advanced (after 5-6 days) the epithelium is largely destroyed and the specimen may consist mostly of dead cells, debris and contaminations.

The general appearance of a living cell under dark ground illumination is already well known (fig. 1). The cell boundary usually shows as a thin bright line, whilst the cytoplasm has a greyish blue diffraction colour probably due to the extreme thinness of its constituent elements, which show as an indistinct network interspersed with small ill-defined granules. The nucleus appears as a dark, more or less clear area which tends to become more distinct with the death of the cell. Unless the cell walls are relatively close to it, the nucleus may be almost entirely obscured by the surrounding cytoplasm. Cell granules, fat, pigment (fig. 2), mitochondria, Golgi apparatus and other intracellular structures are readily visible with dark ground illumination.

Vaccinia virus can be recognised in the corneal cells as early as 24 hours after inoculation. The number of infected cells and the amount of virus seen increases directly with the duration of the infection. In resistant animals the infection is transient and the number of infected cells small. If the preparation shows a sheet of cells the infected area can easily be found by its brightness, which makes it stand out from the dark blue normal

tissue. Figs. 3-6 show the same field at 60, 300, 1000 and 2000 diameters. The normal tissue appears dark in each photograph, the infected area white. In fig. 6 two small spheres are shown in the same field.

From observations on preparations made at different intervals after inoculation and from observations on the changes taking place in a preparation over a period of several days, the following appearances have been recorded.

1. The whole surface of the cell may be studded with elementary bodies which are immobile and appear to be adherent to the gel of the cytoplasm (figs. 3 and 7). At a later stage these single elementary bodies and discs appear to be in oscillating movement, apparently the beginning of liquefaction of the surrounding cytoplasm into which they have sunk.

2. A collection of elementary bodies in rapid Brownian movement may be found in any part of the cytoplasm (fig. 9). This appearance gives the impression of an area of liquefaction in which the elementary bodies are no longer restrained by the gel of the cytoplasm. This structure sometimes closely resembles the Guarnieri inclusion bodies so familiar in stained preparations, more especially when a boundary zone between the gel-like cytoplasm and the more liquid area of the inclusion can be distinguished under favourable conditions.

3. The inclusion bodies are irregular in form but are usually adapted to the shape of the cell. In the flat cells of the surface epithelium the inclusions are of necessity attenuated, whereas in the plumper polygonal cells they are more rounded and compact and frequently crescent-shaped.

4. A cell may contain several inclusion bodies of different sizes or the entire cytoplasm may appear to be hollowed out and filled with rapidly dancing elementary bodies. The smallest inclusion encountered contained a single elementary body (fig. 10), but the average, which is about the size of the nucleus, contains as many as 150 elementary bodies, though their active movement makes counting unreliable.

5. A cell may contain both types of elementary bodies, those stuck to the surface of the cytoplasm and those in rapid movement within an inclusion body.

6. Not only have single elementary bodies been observed, but under favourable conditions discs and spheres have been resolved (fig. 9). Discs were observed immobile in the cytoplasm as well as motile within the inclusions; spheres have also been seen in the inclusions and free in the medium outside the cells. These discs and spheres are similar to those previously described in observations on the life-cycle of pleuropneumonia (Merling-Eisenberg, 1935b).

7. Inclusion bodies are found less frequently in the long fibres of the corneal connective tissue than in the polygonal epithelial cells.

Small fat or lipid droplets are the only intracellular structures likely to be confused with virus elements, but since they are always immobile, vary widely in size and show a more brilliant and yellowish diffraction colour, they may readily be distinguished after some experience. Occasionally large and small pigment cells are found which contain a varying number of pigment particles. These are always immobile, of a yellowish diffraction colour and oblong shape and usually measure $0.3 \times 0.5 \mu$. They show up very brightly and once recognised cannot be mistaken for virus bodies (fig. 2). Mitochondria may be the same size as virus bodies but their brightness when measured photometrically was always found to be one-fifteenth of that of the virus. The Golgi apparatus appears as an ill-defined network of the same brightness as the virus.

Frequently in the early stages the virus appeared only in one part of the lesion while in other parts, *e.g.* in the deeper layers of the cornea, only mitochondria were found. This conveyed the impression that, previous to the appearance of the virus, the cells surrounding the wound of inoculation generally suffer some sort of disintegration which shows itself in the greater number of mitochondria and other granules inside and outside the cells. The mitochondria are capable of being reabsorbed and so the curious picture is produced that, where at first only mitochondria were seen, later only virus may be found. All intermediate stages between these extremes have been observed.

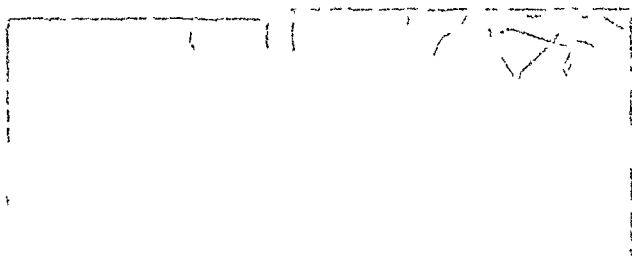
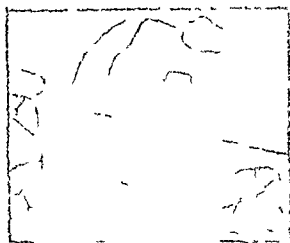
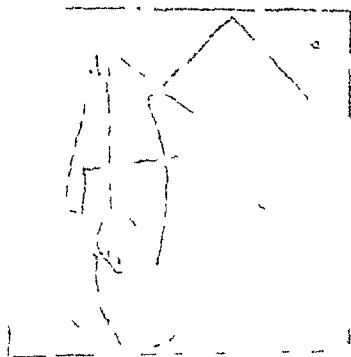
Under the conditions of the experiment the vaccinia virus bodies were too large to show any specific diffraction colour and appeared nearly white. Inclusion bodies sometimes showed a light blue colour. At first the elementary bodies gave the impression of fairly uniform size (fig. 8), but after some experience had been gained, a sequence of sizes could be distinguished from the smallest elementary bodies to the larger disc forms.

Measurements. It was possible to take measurements of inclusion bodies and spheres within the living cell, with the following results :—

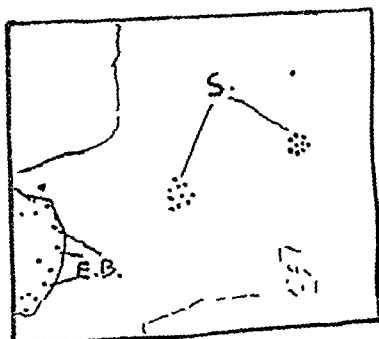
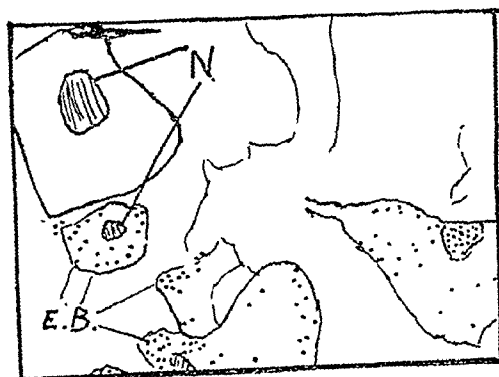
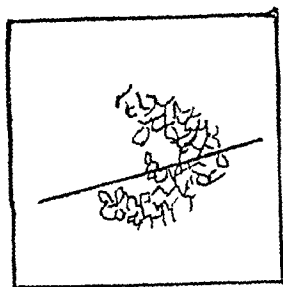
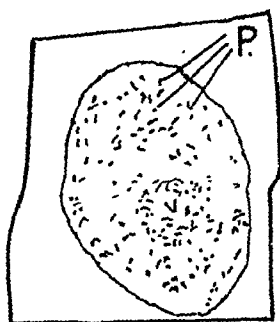
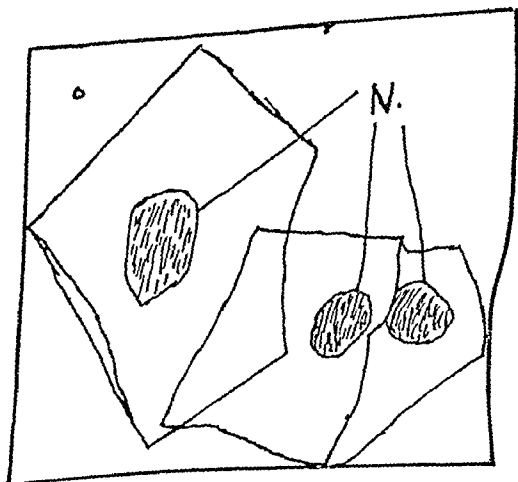
Inclusion bodies	.	.	0.5 to 15.0 μ
Spheres	.	.	0.5 to 1.0 μ

Owing to the limitation of resolution by the watery medium, accurate measurements of elementary bodies and discs cannot be made, but if the following procedure is carried out, the obscuring elements of the cells may be optically extinguished, leaving the virus particles unaffected and still further improving the degree

1 2 1 2 1 1 1



LIVING VACCINIA VIRUS



N. = Nucleus
P. = Pigment

E.B. = Elementary bodies
S. = Spheres

Line through figs. 3 and 4 divides
normal tissue (above) from
lesion (below).

LIVING VACCINIA VIRUS



FIG. 1.—Normal cells. $\times 1200$

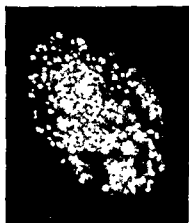


FIG. 2.—Pigment cell. $\times 1200$.

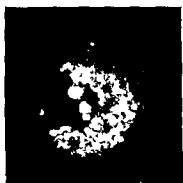


FIG. 3.—Shaving through lesion; 48 hours after inoculation. $\times 60$.

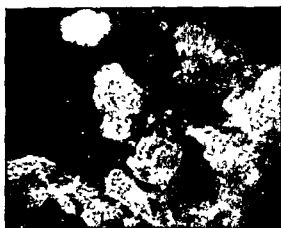


FIG. 4.—The same field as fig. 3. $\times 300$.



FIG. 5.—The same field as fig. 3. $\times 1000$.

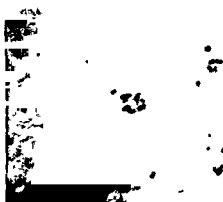


FIG. 6.—The same field as fig. 3, showing 2 spheres. $\times 2000$.

of resolution. The coverslip is removed and the cells allowed to dry on to the slide. A medium of higher refractive index (oil of bergamot, $n_D = 1.46$) is placed on them, with a new coverslip (Merling-Eisenberg, 1935a). Since the oil is of nearly the same refractive index as the cell envelope and cytoplasmic structures, these no longer diffract the light, but the virus particles being of slightly different refractive index stand out clearly. Under these conditions many of the elementary bodies which in the watery medium appear as points of even brightness now appear as rings with a well defined and perfectly resolved dark centre. In the wall of some of these rings from 1 to 5 nodes can be distinguished. By this procedure the following additional measurements were obtained :—

Elementary bodies	.	.	80 to 120 $m\mu$,
Discs	.	.	150 to 200 $m\mu$.

These measurements confirm earlier findings (Merling-Eisenberg, 1937b). Spheres can be recognised, but only with difficulty, since they become flattened out during the process of drying. Similarly inclusion bodies seem to collapse, making it extremely difficult to distinguish them in the oily medium.

Life cycle of vaccinia virus. Although it has not been possible to observe the complete development of an inclusion body from a single elementary body, what are regarded as the earlier stages of this development have been recorded by prolonged observation of a cell infected with only one or two elementary bodies. Under favourable conditions a single elementary body has been seen to develop into a disc at the periphery of which nodes appear. These form into separate elementary bodies and are released into the now liquefied interior of the disc. In this way a small inclusion body has been building. After some time, discs can be seen within the inclusion body and later on spheres can be distinguished by the appearance of small clusters of elementary bodies hanging together and preserving their spherical arrangement. Casagrandi had already suggested in 1910-11 that there might be two forms of the virus, owing to its different behaviour before and after filtration.

During prolonged observations over periods up to 9 days the following stages in the development of the virus were seen.

1. Elementary bodies appeared to adhere to the surface of the cell and were then seen to sink into the cytoplasm within a small liquefied area, where they began to show active Brownian movement.
2. These small liquefied areas, each containing elementary bodies, were seen to coalesce, thus giving the appearance of the Guarneri inclusion body.

3. The elementary bodies contained within the inclusion increased in number. This was probably brought about by the same sequence of events as that observed with pleuropneumonia (Merling-Eisenberg, 1935*b*), since discs and spheres were frequently seen in the inclusions (fig. 13).

From previous experience in the study of other viruses, especially pleuropneumonia, it seems reasonable to assume that the develop-

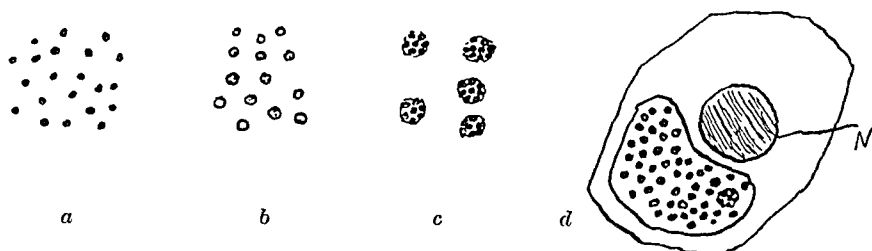


FIG. 13.—Life cycle of the vaccinia virus. (a) Elementary bodies. (b) Discs. (c) Spheres. (d) Virus colony in corneal cell. $\times 1500$.

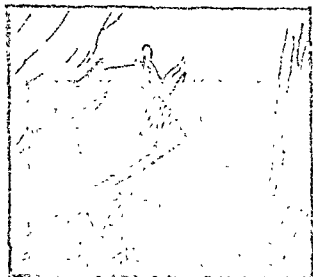
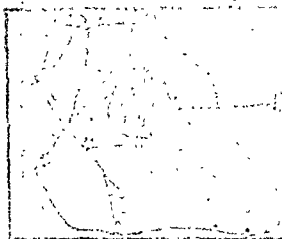
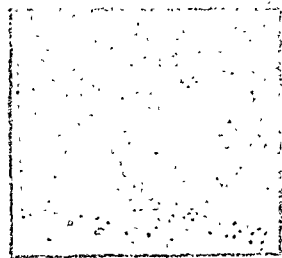
ment of vaccinia virus from the elementary body to the sphere takes place within the infected cell in a regular manner. The final stage is represented by rupture of the cell with discharge of the contents of the inclusion body into the surrounding medium, when the cell collapses and disintegrates into a shapeless mass of granules. This has been observed on several occasions.

Discussion

After consideration of the observations described above there is little difficulty in assuming that the inclusion bodies in the living cell represent the Guarnieri bodies of fixed and stained preparations. Since it has been shown that the living inclusion body has two component parts—the virus bodies and the surrounding liquefied cytoplasm—it is not difficult to account for the different appearances produced by the use of stains and fixatives. Hæmatoxylin and eosin stain the whole structure and the shrinkage of the coagulated fluid portion produces the well known halo effect. Ford's (1934) modification of Mann's stain produces pictures of Guarnieri bodies containing groups of elementary bodies. The time of appearance of inclusion bodies was found by Taniguchi *et al.* (1934) to be 8-10 hours after inoculation. Rhodes and van Rooyen (1937 *a* and *b*) found inclusion bodies as well as elementary bodies after 48 hours.

Barnard (1932) gives photographs of vaccinia virus obtained by means of his ultra-violet light method and the ring forms depicted closely resemble the discs seen during these observations on living virus.

PLATE XXVI

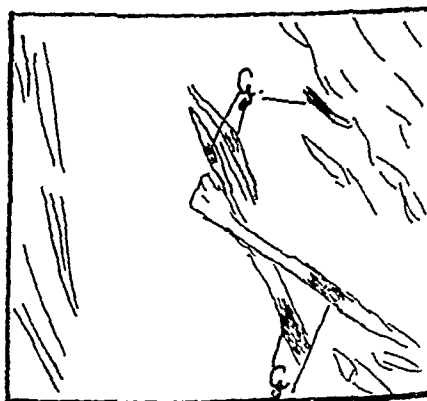
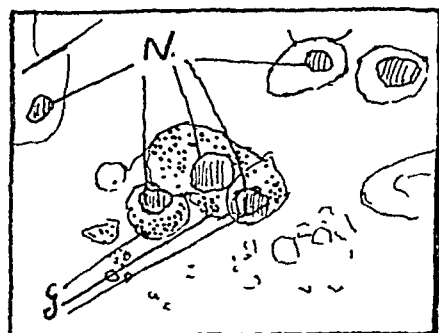
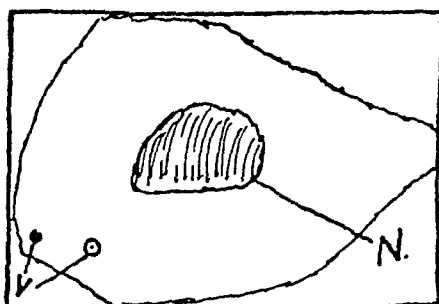
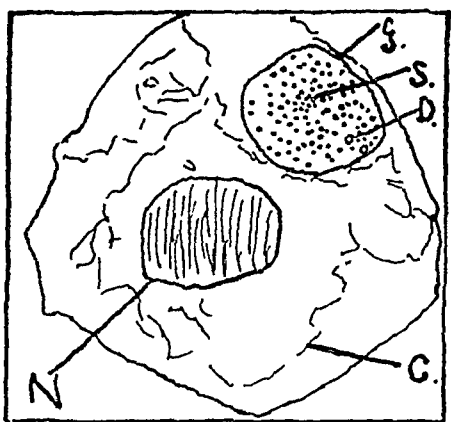
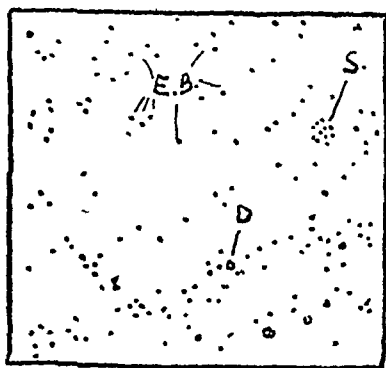
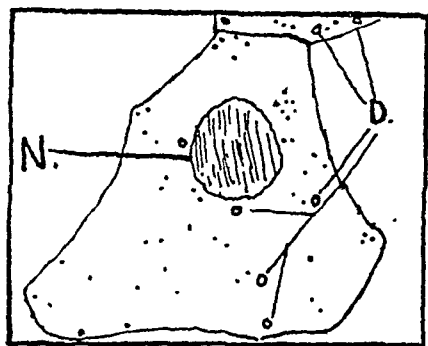


Goodrich - 1901
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LIVING VACCINIA VIRUS



N. = Nucleus
C. = Cytoplasm

E.B. = Elementary bodies
D. = Discs
S. = Sphere

G. = Guarnieri body
V. = Virus colony

LIVING VACCINIA VIRUS



FIG. 7.—Cell studded with virus.
× 1200.

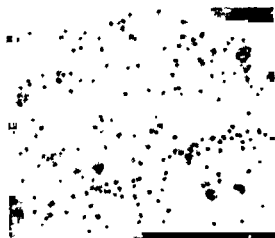


FIG. 8.—Virus free in the medium.
× 1200.



FIG. 9.—Cell with virus colony. × 2000.



FIG. 10.—Cell with 2 small inclusions.
× 1200.



FIG. 11.—Cells studded with virus, containing inclusion bodies. × 600.



FIG. 12.—Connective tissue cells containing inclusion bodies. × 600.

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THE BACTERIOLOGICAL EXAMINATION OF WATER SUPPLIES

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BARDSLEY (1938), following the work of Wilson *et al* (1935), has reported very favourably on the examination of water by a method which involves the usual inoculation of various amounts into MacConkey's medium at 37° C. and subculturing from tubes showing acid and gas within 48 hours to MacConkey's medium (incubated for 48 hrs at 44° C.) and to liquid citrate medium (incubated at 37° C for 2 days). Growth at 44° C. with production of acid and gas is regarded as indicative of the presence of *Bact. coli* and visible growth in citrate medium as evidence of the presence of members of the intermediate *aerogenes cloacae* group (I A C). The method has obvious advantages in respect of cost and speed and has received partial endorsement from the Ministry of Health (Report, 1939). We record herein our experience of this method in the examination of water in this laboratory.

The presumptive coli test

One of the disadvantages of the more elaborate methods of differentiation introduced in recent times into the technique of bacteriological control of water supplies is the delay occasioned in arriving at a final report. Simplicity and speed are of the highest importance provided that the results are not misleading. We have therefore summarised our experience of the simple presumptive test for *Bact. coli* in the past two years in routine work. Most of our samples have been obtained from the taps, pipes and reservoirs of the Liverpool supply, which is derived from upland sources and filtered but in the main is not chlorinated. Some have come from a number of neighbouring supplies. In one series 1127 tubes showed acid and gas—at least sufficient gas to fill the rounded end of the Durham tube—in 18-24 hours. Of these, 947 (84 per cent.) were proved to contain *Bact. coli*. In the whole investigation we have obtained *Bact. coli* from 1252 tubes showing acid and gas within 48 hours; of these 1183 (94·5 per cent.) produced acid and gas within 24 hours and further incubation added 69 (5·5 per cent.). It appears that this simple short test somewhat

exaggerated the frequency of *Bact. coli*—by 16 per cent.—and that only 5.5 per cent. more strains were revealed by prolonging the incubation to 48 hours. Even this slight gain is, we think, slightly exaggerated, for 16 of the 69 additional strains were obtained from 3 samples. The precise time of incubation ranged from 18 to 24 hours according to the time of arrival of the sample and in times of rush tended to be rather short. In waters collected from taps in the laboratory, where the incubation was more regularly 24 hours before the first scrutiny, the strains of *Bact. coli* totalled 374 and of these 360 (96.3 per cent.) produced acid and gas in the presumptive test within 24 hours. The 24-hour presumptive test is therefore a reasonably satisfactory guide to the *Bact. coli* content of the waters which we have examined. For the detection of members of the I.A.C. group the longer incubation is necessary, as a proportion—in some waters a large proportion—of these strains do not produce acid and gas in 24 hours.

Differentiating tests

On applying Wilson's technique to the differentiation of coliform strains producing acid and gas in the presumptive test we found a surprisingly large number of instances in which growth occurred both at 44° C. and in citrate at 37° C. We therefore investigated the specificity of these two tests further.

Growth in MacConkey's medium at 44° C. A loopful (about 2 mm.) of the contents of the tube showing acid and gas in the presumptive test was inoculated into about 5 c.c. of MacConkey's medium previously warmed to 44° C. and incubated in a closed water bath regulated to 44° C. \pm about 0.5° C., readings of acid and gas formation being made at 24 and 48 hours. Most of our strains of *Bact. coli* produced acid and gas in 24 hours but a few required 48 hours. In all, 137 tubes so inoculated and incubated showed acid and gas. Three were not examined further, 133 yielded typical *Bact. coli*—130 in pure culture, 2 with non-lactose-fermenting organisms and one with *Bact. aerogenes*—while one tube contained only *Bact. aerogenes*. The test is therefore of great value in determining the presence of *Bact. coli*. There do however appear to be certain strains of *Bact. aerogenes* which can produce acid and gas in MacConkey's medium at 44° C. In this particular series there were 2 such strains; in a further study of a large number of coliform organisms we met with 11 others. They seemed to be associated with certain sources of supply. Our view is that for the waters which we have had to test in Liverpool the test is as satisfactory a means of detecting *Bact. coli* as could well be devised. In India it has proved much less satisfactory (Raghavachari and Iyer, 1938-39). In distinguishing the unusual *aerogenes* strains we have found the colonial appearances on eosin-methylene blue

plates of great help, as they form large mucoid colonies readily distinguishable from those of *Bact. coli*, and it is now our practice to supplement the test by inoculating a segment of such a plate with a loopful of the contents of the positive tube in the presumptive test. The ordinary strains of *Bact. aerogenes* are so intolerant of the temperature of 44° C. that inocula of 900,000 to 4 million organisms failed to grow (0.1 c.c. of a 1:10 dilution of broth or peptone water culture), whereas the exceptional strains, like *Bact. coli*, developed from minimal inocula—10-50 organisms. With *Bact. coli* the inocula from cultures required to initiate growth at 44° C. in MacConkey's medium in some experiments were identical with those required at 37° C. but in a few they seemed to be larger. Our experiments were not however sufficiently extensive on this point.

In view of the selective nature of incubation at 44° C. we have used it in a series of examinations for the presumptive test in parallel with the usual incubation at 37° C. Altogether 329 samples were tested immediately after collection from the supplies without refrigeration with the following results.

Samples tested	329
<i>Bact. coli</i> absent in both tests	194
No. of <i>Bact. coli</i> the same in both tests .	55
Discrepancies between the results of the two tests	80

The following are the details of the discrepancies.

- (a) *Bact. coli* present at 37° C., absent at 44° C. 31 samples.

The count at 37° C. was 1 in 22 samples, 3 in 8 samples and 5 in 1 sample.

- (b) *Bact. coli* absent at 37° C., present at 44° C. 22 samples.

The count at 44° C. was 1 in 14 samples, 2 in 2 samples, 3 in 4 samples and 5 in 2 samples.

- (c) Count at 37° C. higher than at 44° C. 15 samples.

No of samples	Count of <i>Bact. coli</i> at	
	37° C.	44° C.
2	2	1
7	3	1
2	5	1
1	5	3
1	8	1
1	13	3
1	13	5

(d) Count at 44° C. higher than at 37° C. 12 samples.

No. of samples	Count of <i>Bact. coli</i> at	
	37° C.	44° C.
6	1	3
1	2	3
2	1	5
1	5	8
1	8	11
1	8	25

The counts recorded are the most probable numbers as calculated from McCrady's table (Report, 1939). Most of the discrepancies are of the order that might be expected from the examination of two samples by the same method but some are rather greater. Further study of the presumptive test at the higher temperature would appear to be desirable.

Growth in liquid citrate medium at 37° C.

According to Bardsley's suggestion the occurrence of growth in citrate medium on inoculation with a very small amount of the contents of a positive tube in the presumptive test would be taken as indicating the presence of organisms of the I.A.C. group. In her series of 81 samples of water 8 yielded only organisms other than I.A.C. They were non-lactose-fermenting organisms which were unable to grow in citrate when subsequently tested in pure culture. She suggested that the original growth may have been due to the use of too large an inoculum which carried over utilisable organic matter from the original tube. In the Report of the Ministry of Health it is recommended that a straight wire and not a loop should be used for inoculating citrate media.

We have examined 539 tubes of citrate showing a visible amount of growth within 1-3 days after inoculation from positive tubes in the presumptive test, usually with a straight wire but sometimes with a loop of about 1 mm. diameter. The growth in citrate was plated on eosin-methylene blue agar and representative colonies were picked off at 24 or 48 hours and tested for lactose fermentation, indole production, by the Voges-Proskauer (Barritt's modification) and methyl red tests and for growth in citrate at 37° C. and in MacConkey's broth at 44° C., all results except growth at 44° C. being recorded at 3 days. In 21 tubes the organisms were not identified; in the remainder (518) the following were identified fully or in part.

<i>Bact. coli</i>	183 (28 in pure culture)
I.A.C. group	238
Late lactose fermenters	139 (53 in pure culture)
Non-lactose fermenters	176 (46 not tested in liquid medium; 26 unaccompanied by any other Gram-negative bacillus)

The presence of *Bact. coli* in these tubes was at first surprising but in all cases they had been revealed in the presumptive positive tube by the test at 44° C. None produced visible growth in citrate when subsequently tested in pure culture, whether inoculated with a straight wire or a loop. Their presence ought not to have caused surprise, for Ruchhoft *et al.* (1931) have shown that inocula as small as 1 organism may increase in this medium to about 5 million per c.c. We have confirmed this finding with a number of strains and have shown further that the addition of a loopful of bile broth or peptone water to the citrate does not materially affect the result. In those instances in which *Bact. coli* alone was found the turbidity may well have been due to organisms other than members of the genus *Bacterium*, with which we did not concern ourselves.

The strains of the I.A.C. group were quite typical.

The organisms classified as *late lactose fermenters* failed to ferment lactose in a liquid medium in 24 hours in the routine test and in 3 days produced acid only. On eosin-methylene blue agar (E.M.B.) they produced colonies which were pale at first and later developed dark centres; no metallic sheen appeared and the colonies were not mucoid. Bardsley (1934) showed that a proportion of intermediate (11 per cent.) and *aerogenes* (6 per cent.) strains gave delayed fermentation of lactose, so that our strains might well be properly included within the I.A.C. group. Fifty-two were subsequently examined more fully; 13 now produced acid and gas from lactose within 3 days and 32 within 10 days at 37° C.; 3 others failed to do so at 37° C. but did at 22° C. Probably all should properly be placed in the I.A.C. group, for there is much evidence that its members are more active at temperatures below 37° C. The remaining 17 strains produced acid from lactose in 3 days but failed to produce gas in 10 days. It seems doubtful if such organisms as these should at present be given the sanitary importance of the I.A.C. group. Their reactions in the standard tests were somewhat variable though all grew well in citrate. The majority failed to ferment inositol, dulcitol or salicin but fermented glucose, mannitol, maltose, sucrose and cellobiose. None formed indole or grew at 44° C. or liquefied gelatin.

On 176 plates of E.M.B. colonies of *non-lactose fermenters* appeared, usually associated with other Gram-negative rods, but in 26 instances they were not so accompanied. Of these, 130 strains still failed to ferment lactose in liquid media in 3 days at 37° C. in the first routine test. Fifty-nine of them were examined more fully 1-2 months later and 5 fell into the I.A.C. group while 2 produced acid from lactose and were classified as late lactose fermenters. Fifty-two still failed to ferment lactose and continued to do so in subsequent tests. Of these, 7 subsequently proved to be chromogens of one kind or other and 2 failed to grow in citrate.

Forty-three strains remained which grew in citrate and failed to ferment lactose. In many instances the growth in all media and particularly in citrate was very irregular at 37° C. and suggested that this temperature was very near the maximum for these organisms; at 22° C. or room temperature they all grew reasonably well. Thirty-seven strains were M.R.+, V.-P.-, citrate+, indole-, 44° C.-, the remaining 6 giving a positive V.-P. and negative or doubtful M.R. test. Glucose, maltose and mannitol were readily fermented by all, usually with gas production, cellobiose by all but 4 and sucrose by most of the strains, inositol by 15, salicin by 6 and dulcitol by none. Only 2 strains liquefied gelatin. Stuart *et al.* (1938) have described a number of similar organisms and have drawn attention to their resemblance to the tribe *Erwineae* and particularly to organisms which they isolated from plants. Our strains differed from most *Erwineae* in that they failed to liquefy gelatin. We have not finally classified them but it appears clear that they could not be included in the I.A.C. group without unwarranted extension of that group.

In this series therefore 553 coliform organisms other than *Bact. coli* were identified in 518 tubes of citrate medium and showed visible growth within 72 hours. Of these, 238 (43 per cent.) were undoubtedly members of the I.A.C. group. Late lactose fermenters numbered 139 (25 per cent.). Probably something like three-quarters of these should also be considered as belonging to this group, while the remainder are doubtful. Even if all were regarded as I.A.C. only 68 per cent. of the organisms isolated would be included and 32 per cent. could not. Gas formation in the presumptive test was due in almost all cases to either *Bact. coli* or a member of the I.A.C. group. For the former the test of growth at 44° C. was an excellent criterion but the citrate test did not function nearly so well for the I.A.C. group.

The defects of the citrate test do not appear to have been due to defects in the medium. In the routine examination of pure cultures it was perfectly satisfactory and in experiments it was found that typical *aerogenes*, intermediate, late lactose-fermenting and non-lactose-fermenting strains grew from about the same minimal inocula as they did in MacConkey's medium, while *Bact. coli* did not produce visible growth from very large inocula (0.01 c.c. of a fully grown broth or peptone water culture). In case some defect in the medium might have led to failure to isolate members of the I.A.C. group, we examined 168 positive tubes from the presumptive test simultaneously by direct plating on E.M.B. and by inoculation into citrate medium and subsequent plating. On the plates 31 strains were isolated and through citrate 35 strains; the remainder contained only *Bact. coli*. The results indicate that the medium was adequate.

As stated earlier the majority of inoculations were made from presumptive positive tubes to citrate medium with a straight wire but many were done with a small loop (about 1 mm.). Bardsley (1938) has questioned whether the growth of non-lactose-fermenting organisms in her series may not have been due to the use of too large an inoculum. We think that, other things being equal, the use of a straight wire is not so satisfactory as that of a loop, as one feels less certain about what has been inoculated. From each of 242 positive presumptive tubes we inoculated two tubes of citrate medium simultaneously, one with a small loop and one with a straight wire, and examined them up to 3 days for turbidity due to growth. Of these, 230 gave identical results (147+ and 83-). Ten others were positive with the loop and negative with the straight wire and 2 the reverse. Of the former, 3 of the growths contained I.A.C. and of the latter only 1, the turbidity in the remainder being due to some other organism. We think that the size of inoculum had very little to do with the earlier results and that there were in fact in these waters organisms other than those of the I.A.C. group capable of growth in citrate and of very doubtful sanitary significance.

Summary

1. In the examination of waters from the Liverpool area 84 per cent. of tubes showing acid and gas at 24 hours in the presumptive *coli* test contained *Bact. coli*. Incubation for a further 24 hours revealed only 4.5 per cent. more tubes containing this organism.

2. The production of acid and gas within 48 hours in MacConkey's medium at 44° C. proved a very reliable test for *Bact. coli* in these waters, as the number of other organisms reacting in this way was very small.

3. Growth in liquid citrate medium at 37° C. was a much less specific test for organisms of the group intermediate-*aerogenes-cloacæ* (I.A.C.) on account of the frequent presence of non-lactose-fermenting organisms of unknown sanitary significance which can grow under these conditions.

4. Where speed is important a 24-hour presumptive test is not seriously misleading and the confirmatory test at 44° C. for *Bact. coli* provides an economical and reliable method for the routine examination of these waters. The citrate test seems to add little of value. A presumptive test at 44° C. is worthy of further investigation.

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PATHOLOGICAL CHANGES IN THE SEX ORGANS AFTER PROLONGED ADMINISTRATION OF SEX HORMONES TO FEMALE RATS

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(PLATES XXXI-XXXV)

IN our previous papers (Korenchevsky, 1937, Korenchevsky and Dennison, 1935, 1936 *a* and *b*, Korenchevsky and Hall, 1937, 1938, Hall, 1938) we have studied the effects on the histological structure of the sex organs of rats of male and female hormones injected for comparatively short periods of about three weeks

In experiments of longer duration the pathological properties of the hormones should be more clearly revealed. Such investigations with oestrogenic hormones have been undertaken by several workers (see reviews by Kaufmann and Steinkamm, 1936, Mighavacca, 1936, Simonnet, 1937, Korenchevsky and Hall, 1938, Cook, 1939, Lacassagne, 1939). Similar experiments with male hormones, and especially in combination with female hormones, are urgently needed, since both types of hormones are present simultaneously in either sex, and a disturbance of their normal balance is always possible. The existing literature dealing with the effects on females of male hormones alone or in combination with female compounds has been reviewed recently by Korenchevsky (1939). Later papers (Fischer, 1938, Courrier and Gros, 1938 *a* and *b*, Groome, 1939, Silvestroni, 1939) have confirmed the bisexual effects of male hormones and other histological changes induced by them in the sex organs.

References dealing with certain special changes in the sex organs, especially the ovaries, will be mentioned in the text when these changes are described, in order to facilitate comparison and the drawing of general conclusions. Preliminary notes on certain changes obtained in some of the rats discussed in this paper have

* Dr Korenchevsky desires to express to the University of Oxford, to Professor E S Goodrich and to Professor J A Gunn his own and the Lister Institute's gratitude for the kind hospitality extended to him and his co-workers in the Department of Zoology and Comparative Anatomy and the Nuffield Institute for Medical Research.

been given elsewhere (Korenchevsky, Burbank and Hall, 1939; Korenchevsky and Hall, 1939; Korenchevsky, Hall and Burbank, 1939).

Technique. Details of our general technique have been given in our previous papers. The present experiments were performed on 181 rats

TABLE I

Effect of the hormones on the weight of the sex organs of normal (groups 25-30) and spayed (groups 3-11, 14-18, 20-23) rats as compared with those of control rats injected with pure oil (groups 1, 2, 12, 13, 19, 24)

Experiment	Group	Hormone and weekly dose (mg.) injected	Weight in mg. of			No of rats in group
			uterus	vagina	ovaries	
(A) On spayed rats	1	Control normal rats (in diestrus)	448	292	...	3
	2	Control spayed rats	34	190	...	8
	3	Œstradiol D * 0.018	266	332	...	3
	4	Œstradiol D 0.090	283	350	...	5
	5	Androsterone 7.5	59	312	..	4
	6	Androsterone 7.5 + Œstradiol D 0.018	327	416	...	3
	7	Androsterone 7.5 + Œstradiol D 0.09	357	440	..	3
	8	Dehydroandrosterone 7.5	63	253	..	4
	9	Dehydroandrosterone 7.5 + Œstradiol D 0.09	366	366	...	4
(B) On spayed rats	10	Œstradiol BB * 0.09	473	422	...	6
	11	Œstradiol BB 0.09 + androsterone 7.5	536	452	...	8
(C) On spayed rats	12	Control normal rats (in diestrus)	526	324	..	6
	13	Control spayed rats	33	175	..	5
	14	Œstradiol D 0.2	326	311	...	9
	15	Testosterone P * 2.25	262	471	..	7
	16	Testosterone P 7.5	395	534	...	4
	17	Testosterone P 2.25 + Œstradiol D 0.2	511	484	...	6
(D) On spayed rats	18	Testosterone P 2.25 + Œstradiol D 0.2 + progesterone 4.5	337	463	..	9
	19	Control spayed rats	43	214	...	8
	20	Œstradiol BB 0.09	308	373	...	12
	21	Œstradiol BB 0.09 + testosterone P 7.5	411	655	...	6
	22	Œstradiol BB 0.09 + testosterone D 7.5	452	565	...	13
(E) On normal rats	23	Testosterone D 7.5	386	492	..	6
	24	Control normal rats	74	17
	25	Œstradiol BB 0.09	712	483	(57-93)† 63 (23-102)	5
	26	Œstradiol BB 0.09 + androsterone 7.5	940	638	66 (35-97)	2
	27	Testosterone P 2.25	383	522	27 (24-30)	2
	28	Testosterone P 7.5	3295†	611	38 (33-43)	2
	29	Œstradiol BB 0.09 + testosterone P 7.5	1101	597	44 (20-91)	6
	30	Œstradiol BB 0.09 + testosterone D 7.5	977	592	40 (20-52)	5

* The following abbreviations are used for denomination of the esters of the hormones.
P = propionate; D = dipropionate; BB = 3-benzoate-17-n-butyrate.

† Figures in brackets represent range of variation of weights of ovaries.

‡ Uterus was distended with secretion.

of known age and belonging to the same strain, the members of each litter being distributed as evenly as possible between the different groups of each separate experiment. The ages of the rats at the close of the experiment varied from 110 to 214 days, being in most litters about 150 days. The animals were injected for periods varying from 53 to 146 days, the majority of litters for about 3 months. Ovariectomy was performed at the age of 21-23 days. The arrangement of the experiment is shown in table I, which gives the distribution of the rats among the different groups, the hormones and doses injected, and the changes in weight of the sex organs. Groups 25-30 had no controls, the weights given for the ovaries being those of rats from different litters but of similar age. The weights of uterus and vagina of these groups can be compared with those of groups 1 and 12 of a similar age. The organs were fixed in Bouin's fluid and embedded in paraffin. Sections were stained with Ehrlich's hæmatoxylin and eosin and, where necessary, with Masson's trichromic stain for fibrous and muscular tissue and with Mayer's mucicarmine.

GENERAL CHANGES OBTAINED

We propose to describe first the general changes obtained in the sex organs, and then, because of their importance, to give a special description of the metaplastic changes in a separate section. Since in all our publications photomicrographs are always printed at the same magnification for each organ, no photographs of the normal uterus and vagina are given here, but the reader is referred to the figures in our previous publications in this *Journal*, in which the structure of these organs at every period of the sexual cycle or under the influence of different sex hormones is described.

The effects of œstradiol dipropionate or 3-benzoate-17-n-butyrate

These œstrogenic compounds were chosen because their effect is greater and lasts much longer than that of pure œstradiol (Korenchevsky, Burbank and Hall, 1939). Except in one experiment in which 0.018 mg. per week was injected, the doses given were comparatively large—0.09 mg. per week, or, in experiment C, 0.2 mg. per week. The pathological changes and in particular the metaplastic changes in the uterus develop more readily with large doses.

Ovariectomised rats

Uterus. As compared with experiments of shorter duration in which pure œstrone or œstradiol was injected (Korenchevsky and Hall, 1937, table on p. 688 and fig. 63), a larger uterus was obtained with the esters of these hormones (table I of this paper, groups 3, 4, 10, 14, 20) and better development of the myometrium and mucosa (fig. 1). However, in most cases a uterus of normal weight was not obtained, while pathological changes appeared. In addition to metaplasia of the epithelium, the mucosa was more

fibrous and contained fewer uterine glands than normal. In some cases small or medium distension of the uterus was noted, but the full distension found at normal oestrus was never present. The epithelial cells had a structure typical for this hormone (Korenchevsky and Hall, 1937, fig. 83), being abnormally high and columnar; or, in some cases with distension of the uterine lumen, having a "tumbler" appearance with occasional vacuolation of the cells. Except for their much larger size (fig. 10, this paper), these "tumbler" cells were reminiscent of those previously described after injection of a large dose of testosterone propionate (Korenchevsky and Hall, 1937, fig. 81). In some cases after oestradiol injections local patches of oedema appeared in the sub-epithelial stroma, with or without the formation of cysts either between the epithelial layer and the stroma or in the epithelial layer itself. These appeared to be formed often by local accumulation of oedematous fluid between the basal and upper rows of epithelial cells. They were different from the cysts obtained after injections of oestrogens into normal rats or after combined injections with testosterone (*vide infra*), when they are always formed by distension of the uterine glands. With the doses of oestradiol used, the uterine glands in our ovariectomised rats were always few and narrow; with the doses used we have never seen cystic glands in spayed rats. The accumulation of oedematous fluid under the epithelial layer is followed by desquamation of more or less large areas of this layer (fig. 1).

Vagina. In contrast to the uterus, the vagina reached normal or supernormal weight. The histological structure of the vaginal wall was normal, but the epithelial layer showed great variations. It was always well developed, consisting of several rows of large squamous cells with or without varying degrees of keratinisation of the upper rows. In experiments previously described (Korenchevsky, Burbank and Hall, 1939), in which spayed rats were killed on the 106th day after the last injection of oestradiol benzoate-butyrate, varying amounts of mucification were obtained. In one rat the degree of mucification was similar to that found during pregnancy (fig. 11). Until recently mucification has been considered to be the typical reaction of the vaginal epithelium to progesterone and testosterone compounds and in pregnancy, keratinisation being typical for the oestrogenic hormones. In the above-mentioned paper (1939, p. 369), we have suggested a possible explanation of this unusual "oestrogenic" reaction.

Normal rats

Uterus. In the injected non-spayed rats the uterus was larger than in the controls (table I, group 25, and fig. 9), with or without a varying degree of distension of the lumen. The epithelium

consisted of typical high columnar or more rarely "tumbler" cells, as described for ovariectomised rats. In the distended uteri there were small villous projections involving either the epithelial layer alone or the whole of the mucosa; these are an unusual oestrogenic effect and were reminiscent of the abortive lace-like foldings found during pregnancy.

The following changes may be described as definitely pathological features:—(1) An irregular development of the circular layer of the myometrium which was in places oedematous. (2) Cystic enlargement of some of the glands, which, with the doses used, never occurred in ovariectomised rats. The latter fact suggests that some other hormone or hormones are responsible, in addition to oestrogens, for the rapid development of cystic hyperplasia. (3) The uterine stroma was in places more fibrotic than normal, and in the parts adjoining the oedematous muscular layer it was also oedematous.

Vagina. The vagina in all the injected rats was much larger than normal (group 25). The structure was normal, except that instead of the expected keratinisation of the epithelial layer, typical pregnancy mucification was present in half of the rats, slight and partial mucification and swelling of the cells in the others.

Ovaries. Although several workers have investigated the effect of oestrogens on the ovaries, their conclusions have not been unanimous.

Meyer *et al.* (1929 30), Doisy, Curtis and Collier (1930 31), Kunde *et al.* (1931) and Zondek (1936, 1937 *a* and *b*) found that injections of oestrogens inhibit the development of the ovaries, cause a decrease in their size and weight, and in some cases produce degenerative changes. The ovaries are pale in colour, corpora lutea are absent or degenerate, the follicles are small or atretic and the germinal epithelium is in a state of dropical degeneration. In Zondek's (1936) experiments on rats the ovaries of the control animals weighed 37 mg, those of the treated animals 15 mg.

On the other hand Hohlweg (1934), Wolfe (1934 35), Selye, Collip and Thomson (1934-35) and Nelson (1935 36) obtained large ovaries containing large and numerous corpora lutea, the latter exceeding in size those found at oestrus and equal to those of pregnant animals.

These discrepancies have been explained by (*a*) difference in the size of the doses used (Selye, Collip and Thomson) or (*b*) the fact that if the experiments are commenced at a too early infantile age, the ovaries do not respond to the luteinising action of the oestrogens (Hohlweg's experiments on rats, 1934), while later they do. Hisaw, Fevold, Foster and Hellbaum (1934) and Selye *et al.* have suggested that the effect of oestrogens is produced not directly but through a change in the secretion of the luteinising and follicle stimulating hormones of the anterior lobe of the hypophysis.

Apparently conflicting results such as those above mentioned were also obtained in our experiments. Of the five rats, two had large ovaries (102 and 90 mg.) with several enlarged corpora lutea, two had medium sized ovaries (53 and 46 mg.) with corpora lutea

of approximately normal size, while the fifth had very small ovaries (23 mg.) with few and small corpora lutea, only one of which was of nearly normal size. The corpora lutea, however, consisted of apparently normal lutein cells. In all the ovaries most of the follicles were more or less degenerated or atretic, and no large follicles were present. The cytoplasm of the interstitial cells was completely vacuolated; the vacuoles were large and probably dropsical in nature. The contrast between the apparently normal lutein cells and the mostly degenerated follicular and vacuolated interstitial tissue was striking. There was no increase in the amount of fibrous connective tissue.

The first injection of œstradiol in each of our experiments was given simultaneously to each rat in the group and all the rats were killed on the same day. Therefore the differing results obtained could only be accounted for by differences in the rats themselves. Even in two rats from the same litter injected with similar doses of the same compound one had an apparently normal ovary (fig. 14), while in the other (fig. 15) the gland was exceedingly small and atrophic. The injections into these rats were started at 53 days of age, *i.e.* after sexual maturity had been reached. It should be mentioned, however, that two rats with very large ovaries and corpora lutea were about 2 months older than the others.

Taking into consideration our own results and those of other workers, it is reasonable to suggest that the differing reactions obtained may be explained by the fact that the balance of the interaction of the endocrine glands is very probably not the same in all animals. Both in our own experiments and in those of other workers, several examples of important co-operative and antagonistic activity of the hormones have been found, and also, in some cases considerable, physiological variations in the size and weight of the endocrine glands.

Normal or supernormal development of the corpora lutea in the rats injected with œstradiol, resulting in a high output of progesterone, explains at least in part the mucification of the vaginal epithelium. The fact that mucification of the vaginal epithelium occurs also in ovariectomised rats, however, indicates that some other mechanism (for example progesterone from the adrenals—Korenchevsky, Burbank and Hall, 1939) may also be operative.

Summary

1. Although lengthening the period of injections of œstrogens resulted in a greater size and weight of the uterus and vagina in spayed rats, and gigantic size of these organs in normal rats, the pathological changes in the sex organs were also increased.

PLATE XXXI

All doses shown in this and following plates are weekly and in mg.

- FIG. 1.—Oestradiol benzoate-butyrate 0.09 injected into spayed rat. Irregular development of circular myometrium. About $\frac{3}{4}$ of epithelial layer in a state of squamous metaplasia, the other normal $\frac{1}{4}$ (thin) is desquamated. Few small glands in fibrotic mucosa. Uterus 449 mg. Rat 255 g. Period of injections 82 days.
- FIG. 2.—Androsterone 7.5+oestradiol benzoate-butyrate 0.09 injected into the spayed litter mate of the above rat. Better developed uterus and normal myometrium, but metaplasia involves the whole layer and most glands and is of more severe degree. Uterus 510 mg. Rat 330 g. Period of injections 82 days.
- FIG. 3.—Testosterone dipropionate 7.5+oestradiol dipropionate 0.09 injected into spayed rat. Normal myometrium, cystic hyperplasia of mucosa ("Swiss cheese" mucosa), epithelial layer of columnar but vacuolated cells. Uterus 402 mg. Rat 235 g. Injections 64 days.
- FIG. 4.—The same injections into spayed rat. The whole epithelial layer is in a state of metaplasia with commencing leucoplakia. Uterus 698 mg. Rat 250 g. Injections 110 days.
- FIG. 5.—Testosterone propionate 7.5+oestradiol benzoate-butyrate 0.09 injected into spayed rat. Uterine lumen in centre, lined with high columnar epithelium. On the left, adenomatous network; on the right, large and small cysts. Uterus 351 mg. Rat 305 g. Injections 53 days.
- FIG. 6.—Testosterone dipropionate 7.5+oestradiol dipropionate 0.09 injected into spayed rat. On right side, part of uterine epithelial layer and nearly all adenomatous network in a state of squamous metaplasia. Uterus 387 mg. Rat 220 g. Injections 65 days.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.

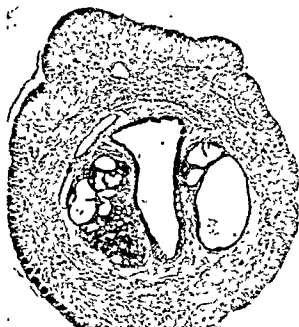


FIG. 5.



FIG. 6.

2. The most important of these changes in the uterus were metaplasia and desquamation of the epithelium and increased amount of fibrous tissue in the mucosa in all rats; the appearance of subepithelial oedema and epithelial or subepithelial cysts in spayed rats and of glandular cysts in normal rats; and irregular development and oedema of the circular muscle layer in normal rats.

3. In all normal rats the vaginal epithelium was mucified in different degrees, sometimes as much as during pregnancy; in spayed rats mucification did not appear until 3-4 months after the last injection of oestradiol benzoate-butyrate was given.

4. In most of the rats the ovaries were enlarged or of normal size and weight, with enlarged or normal corpora lutea; in one rat the ovaries were very small, with few and mostly small corpora lutea. While the corpora lutea appeared to be of normal structure, the follicles were of small or medium size and most of them were atretic or degenerated. The cells of the interstitial tissue were vacuolated.

Androsterone alone or with oestradiol esters

Ovariectomised rats

Uterus and vagina. As compared with the previous experiment of shorter duration (Korenchevsky and Dennison, 1936a), prolongation of the period of injections of androsterone alone definitely improved the development of the vagina but not of the uterus, which was atrophic (table I, group 5) and of a structure similar to that described before. The vagina reached normal size and weight, but although 4-6 rows of cells constituted the epithelial layer, these cells were atrophic and less regularly arranged than in the normal control rats.

The addition of oestradiol produced a marked co-operative effect with androsterone on the better development of the sex organs, in particular of the myometrium (table I, groups 6, 7 and 11 as compared with 3, 4 and 10, also fig. 2 as compared with fig. 1). Certain pathological features were present, however: in the uterus, in addition to metaplasia, a slightly increased amount of fibrous tissue in the mucosa; in the vagina, dropsical vacuolation of the otherwise well developed epithelial layer, the cells of which were swollen, lightly stained and in some cases contained a few droplets of mucin; in other rats the effect of the oestradiol (keratinisation of the epithelial layer) was predominant.

Normal rats

The size and weight of the uterus and vagina were much greater after injections of both androsterone and oestradiol than after

œstradiol alone (groups 26 and 25). Certain pathological features of the type described in normal rats injected with œstradiol alone were present.

Uterus. This showed an increased amount of fibrous tissue in the mucosa and myometrium and partial metaplasia of the epithelial layer, which otherwise consisted of high columnar cells, some of them with dropsical vacuolation. Glands were comparatively few and some were moderately cystic.

Ovaries. These varied, as in the rats injected with œstradiol alone; in one animal they were large (97 mg.) and contained large corpora lutea (fig. 16), in the other (fig. 17) they were small (35 mg.) with atrophic corpora lutea and with small and a few medium sized follicles. Several of the latter were in a state of atresia and in process of transformation into interstitial cells. The greater part of the atrophic ovary was composed of interstitial tissue, with no increase in the amount of fibrous connective tissue. Androsterone alone, according to Hohlweg (1937), did not produce luteinisation of the ovaries of immature rats.

Vagina. It is noteworthy that the vaginal epithelium in the first rat was completely mucified, probably with the co-operation of progesterone from the large corpora lutea, while in the rat with atrophic corpora lutea it consisted of swollen cells with slight keratinisation of the upper rows.

Summary

1. Lengthening the period of injections of androsterone into spayed rats was followed by better development of the vagina but not of the uterus.

2. The administration of androsterone in addition to œstradiol caused a co-operative stimulating effect on the uterus and vagina of both spayed and normal rats, in the latter producing gigantism of these organs.

3. When both hormones were injected, the vaginal epithelium of normal rats became mucified or swollen, while dropsical vacuolation was usually present in spayed rats.

4. The ovaries could be either large or atrophic and contain large or atrophic corpora lutea.

Trans-dehydroandrosterone alone or in combination with œstradiol esters

Ovariectomised rats

Uterus and vagina. The data are given in table I, groups 8 and 9. In spite of prolonging the period of injections, the restorative effect of the hormone remained small (group 8) and the histological changes were similar to those previously described (Korenchevsky

THE STATE

The State of New York, in and for the County of Albany, do hereby certify that the following is a true and correct copy of the original of the same, as the same appears from the records of the said County of Albany, to wit:

That the said original is a true and correct copy of the original of the same, as the same appears from the records of the said County of Albany, to wit:

That the said original is a true and correct copy of the original of the same, as the same appears from the records of the said County of Albany, to wit:

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PLATE XXXII

- FIG. 7.—Testosterone dipropionate 7.5+œstradiol benzoate-butyrate 0.09 injected into spayed rat. Metaplasia (with leucoplakia in places) of epithelium of lumen, large cysts; below left, beginning of adenomatous growth. Patches of cellular infiltration in mucosa. Uterus 544 mg. Rat 270 g. Injections 64 days. $\times 24$.
- FIG. 8.—The same injections into spayed rat. From right to left, huge cyst, uterine lumen with epithelial metaplasia, network of commencing adenoma. Uterus 439 mg. Rat 260 g. Injections 64 days. $\times 24$.
- FIG. 9.—œstradiol benzoate-butyrate 0.09 injected into normal rat. Circular myometrium irregular with (at top) œdema and increased fibrous tissue. Glands partly cystic; mucosa fibrotic. Uterus 908 mg. Rat 265 g. Injections 53 days. $\times 24$.
- FIG. 10.—œstradiol dipropionate 0.09 injected into spayed rat. Very high tumbler-like epithelial cells of uterine mucosa, with occasional vacuolation. Uterus 229 mg. Rat 175 g. Injections 124 days. $\times 200$.
- FIG. 11.—Spayed rat killed on 106th day after last injection of œstradiol benzoate-butyrate 0.09. "Pregnancy" mucification of vaginal epithelium. Vagina 323 mg. Rat 285 g. Injections 21 days. $\times 210$.

UTERINE HORN AND UTERINE AND VAGINAL MUCOSA



FIG. 7.

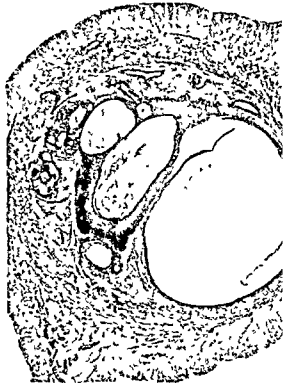


FIG. 8.



FIG. 9.





and Hall, 1937). Both uterus and vagina were atrophic. Mucification of the vaginal epithelium was again found and was more pronounced than that shown in fig. 34 of our previous paper (Korenchevsky and Hall, 1937).

Simultaneous administration of dehydroandrosterone with œstradiol (group 9) was followed by slightly better development of the uterus than with œstradiol alone (group 4), but not of the vagina. In both organs the histological picture was typical for œstradiol alone and the mucification of the vaginal epithelium was replaced either by keratinisation or the formation of a layer of clear, swollen, squamous cells.

Summary

1. Lengthening the period of the injections did not strengthen the effect of dehydroandrosterone on the sex organs of spayed rats.

2. When œstradiol and dehydroandrosterone were injected simultaneously, the effect of the œstradiol predominated, with disappearance of the mucification of the vaginal epithelium, which appeared after and was typical for dehydroandrosterone alone.

*Testosterone esters alone or with œstradiol esters,
or with the latter+progesterone*

Ovariectomised rats

Uterus. After long periods of injections of testosterone propionate or dipropionate, no essential differences were found as compared with experiments of shorter duration (Korenchevsky, Dennison and Eldridge, 1937; Korenchevsky and Hall, 1937; Korenchevsky, Hall, Burbank and Ross, 1939), except for a greater enlargement of the uterus (table I, groups 15, 16 and 23). As in the previous experiments, the pathological changes included fibrosis in the mucosa, an increased amount of fibrous tissue in the myometrium, cellular infiltration of the subepithelial mucosa, enlarged uterine glands and slightly developed lace-foldings of the mucosa (Korenchevsky and Hall, 1937, figs. 59 and 61). These foldings, but much better developed, are typically present during pregnancy and after administration of progesterone in normal rats, or progesterone and small doses of œstrogens in spayed rats.

Important additional changes appeared, however, when *testosterone compounds and œstradiol esters* were injected simultaneously. Generally with, sometimes without, metaplastic changes (*vide infra*), a very pronounced cystic hyperplasia of the uterine glands developed—a typical “Swiss cheese” mucosa, similar to the cystic glandular hyperplasia which occurs in the uterine mucosa of women (*c.g.* see Kurzrok, 1937). Fig. 3 shows this change in a rat's uterus without metaplasia, the epithelium being vacuolated. Silvestroni

(1939) has also described vacuolation of the epithelium after similar injections. In some cases the cysts were so large that they occupied the greater part of the mucosa in cross section and had a diameter equal to or much greater than that of the uterine lumen (figs. 7 and 8).

A similar cystic hyperplasia was produced by the administration of very large doses of oestradiol alone in an ovariectomised woman (Kaufmann, 1933-34, 1935) and in spayed rats (Kaufmann and Steinkamm, 1936; Silvestroni, 1939). Kurzrok (1937, p. 354) considers this hyperplasia to be due to "a dysbalance between follicular hormone and progesterone in favor of the former." Our own results, however, suggest that this clinical picture is more probably, or at least in some cases, due to a simultaneous over-secretion of oestrogens and some male hormone, since in our experiments we have never been able with the doses used to produce it in spayed animals with oestrogenic compounds alone.

In addition to the appearance of metaplasia and a cystic hyperplasia, simultaneous administration of compounds of testosterone and oestradiol also produced another important abnormality—branching of the tubules of the uterine glands, in some cases developing into adenomatous growth (figs. 5, 6, 7 and 8).

It is remarkable that in spite of the considerable pathological changes in the uterine mucosa, the myometrium was in most cases more normally developed than with oestradiol alone (fig. 1). The increase in the size and weight of the uterus was greater when the two hormones were injected simultaneously (table I, groups 17 and 22 as compared with groups 14 and 20), thus demonstrating another useful co-operative activity of the two hormones.

When *progesterone* was injected in addition to *oestradiol* and *testosterone propionate* (group 18) a smaller uterus was obtained and the cysts and metaplastic changes were few or absent (antagonistic effect of progesterone). The uterine epithelium of these rats was vacuolated (as in fig. 3), with or without slight lace-like foldings. The absence of well developed lace foldings typical for progesterone must be explained by the antagonistic effect of the large doses of oestradiol used (Korenchevsky and Hall, 1937).

Vagina. This was larger than normal, both after the injection of testosterone compounds alone or these in combination with female hormones. In experiment D these two groups of hormones had a definite co-operative effect on the development of the vagina (groups 21 and 22 as compared with groups 20 and 23). Because of the large doses of the oestradiol esters used, in the progesterone-injected group 18 the structure of the vagina was more typical for oestrogenic hormone, with a hypertrophic epithelial layer of squamous cells with or without keratinisation. When there was no keratinisation, the squamous cells were clear and swollen or

INDEX

1. Introduction 1

2. Preliminary Results 2

3. Main Results 3

4. Conclusions 4

5. Acknowledgments 5

6. References 6

7. Appendix 7

8. Bibliography 8

9. Index 9

10. Summary 10

11. Notes 11

12. References 12

13. Appendix 13

14. Bibliography 14

15. Index 15

16. Summary 16

17. Notes 17

PLATE XXXIII

- FIG. 12.—Normal control rat. Several corpora lutea and follicles of different sizes. Ovaries 60 mg. Rat 250 g.
- FIG. 13.—Testosterone propionate 2.25. Atrophic ovary with small follicles, some of which are degenerating. No corpora lutea. Ovaries 24 mg. Rat 295 g. Injections 119 days.
- FIG. 14.—Oestradiol benzoate-butyrate 0.09. Large and numerous corpora lutea but small follicles. Ovaries 53 mg. Rat 180 g. Injections 53 days.
- FIG. 15.—The same injections. Atrophic ovary, with a few and mostly small corpora lutea and small follicles. Ovaries 23 mg. Rat 180 g. Injections 53 days.
- FIG. 16.—Androsterone 7.5+oestradiol benzoate-butyrate 0.09. Many enlarged corpora lutea, but follicles of medium or small size. Protective stage of the reaction. Ovaries 97 mg. Rat 265 g. Injections 89 days.
- FIG. 17.—The same injections but ovary atrophic, with small and few medium-sized follicles. Few degenerating corpora lutea. Collapse of the protective reaction. Ovaries 35 mg. Rat 266 g. Injections 89 days.



FIG. 12.



FIG. 14.

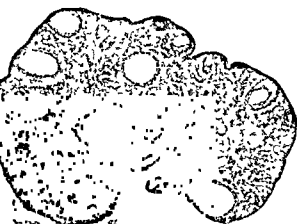


FIG. 13.



FIG. 15.

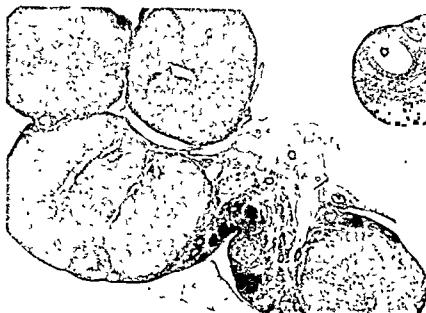


PLATE XXXIII

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partly mucified. With testosterone compounds alone, the same mucification of the vaginal epithelium was obtained as before (Korenchevsky and Hall, 1937, fig. 38 or 41).

Summary. 1. Compared with experiments of shorter duration, lengthening the period of administration of testosterone esters into ovariectomised rats was followed by the development of a larger uterus and vagina, but of a similar pathological structure.

2. After simultaneous injections of testosterone and oestradiol esters, still greater development of these organs was obtained, but additional and sometimes very pronounced pathological changes appeared in the uterine mucosa—cystic hyperplasia of the glands ("Swiss cheese" mucosa), with, in some cases, their adenomatous growth, and more or less general squamous metaplasia of the uterine epithelium.

3. The myometrium, however, after injections of the two hormones, was better developed and of a more normal structure than with either of these compounds alone.

4. While in such cases the changes in weight and size of the sex organs and the development of the myometrium were comparatively constant co-operative effects, any of the changes in the mucosa could appear in different rats alone, or some or all simultaneously, thus demonstrating the resistance or predisposition of individual animals to these substances.

5. The addition of progesterone to male and oestrogenic hormones was followed by the development of a smaller uterus and, because of the large doses of oestradiol used, the absence of pronounced progestational changes in the mucosa.

Normal rats

Uterus. Unexpected results were obtained after prolonged injections of the smaller dose of testosterone propionate (group 27), the uterus being decreased in weight and size as compared with that of normal control animals of a similar age (group 12). The histological structure of the uterus in one rat was similar to that in an adult rat of younger age; in the other there was definite increase of fibrous tissue in the mucosa and myometrium, *i.e.* the effect was similar to, though of less degree than that in ovariectomised animals injected with the same hormone. In our previous paper (Korenchevsky, Hall and Burbank, 1939, p. 379) we explained this small uterus by the depressing effect of testosterone propionate on the secretion of gonadotropic hormone by the hypophysis, the small dose of the hormone being unable to counterbalance this depressing effect produced by direct stimulation of the uterus by the testosterone.

The following facts prove that testosterone compounds have a direct stimulating action on the secondary sex organs, in particular

the uterus, and that they do not act through the hypophysis and ovary only. (1) Testosterone compounds stimulate their development in ovariectomised animals, as was shown previously by ourselves and in the present paper, as well as by other workers. (2) Leonard, Sager and Hamilton (1937-38) found the same effect in hypophysectomised or hypophysectomised and ovariectomised animals.

Mazer and Mazer (1939) injected 30 rats with 0.5 mg. of testosterone propionate three times weekly for 62-102 days and obtained a still more striking decrease in the size of the uterus, which histologically had an atrophic structure. They state that this atrophy was even greater in the rats killed 20 days after the last injection, *i.e.* there was no recovery but, instead, an increase in the atrophying process.

In our experiments with the larger dose (group 28) gigantic uteri were obtained weighing about 2 and 4.5 g. This great weight was partly explained by the fact that the uterine lumen was distended with secretion. Except that the epithelium consisted of columnar cells and that several of the glands in the mucosa were cystic, the structure of the uterine wall was similar to that of an oestrous uterus. A few villous projections were present.

Vagina. On the other hand the vagina showed hypertrophy with both the small and the large doses (groups 27 and 28). Both produced mucification of the vaginal epithelium in different degrees, greater with the large dose, and dropsical vacuolation of the epithelium was also present with the small dose.

Ovaries.

In experiments of not more than 30 days' duration Korenchevsky, Dennison and Hall (1937), Hohlweg (1937), Nelson and Merkel (1937), Wolfe and Hamilton (1937-38), Hamilton and Wolfe (1938, in pregnant rats) obtained large ovaries with enlarged corpora lutea after injections of large doses of testosterone propionate, but the size and weight of the organs were not altered by smaller doses (Korenchevsky *et al.*, Nelson and Merkel). Wolfe and Hamilton obtained large corpora lutea only when the injections of testosterone propionate were started during oestrus; McKeown and Zuckerman (1937-38), on the other hand, could obtain them in rats irrespective of the cyclic period at which the injections were commenced. Aschheim and Varangot (1939) injected adult rats for 5-60 days and obtained hyperaemic ovaries containing normal follicles and corpora lutea. After a single injection, Salmon (1938) in rats and Starkey and Leatham (1938) in mice found follicular stimulation in the enlarged ovaries but no corpora lutea.

McEuen, Selye and Collip (1937), Fischer (1938) and Mazer and Mazer (1939) in rats and Cotte, Martin and Mankiewicz (1937), and Courier and Gros (1938 *a* and *b*) in rabbits, however, after testosterone propionate injections found small ovaries (according to Mazer and Mazer, 3.4 times smaller than those in controls). The histological changes (Cotte *et al.*, Mazer and Mazer) included arrested development of the follicles, absence of corpora lutea and sometimes fibrotic stroma.

PLATE XXXIV

FIG. 18.—Testosterone propionate 10·5, injected for 3 weeks only. Chiefly follicle-stimulating effect—first stage of action of the hormone on ovaries, usually observed (in pure form without corpora lutea) after injections for a few days only. Ovaries 131 mg. Rat 242 g. $\times 18$.

FIG. 19.—Testosterone propionate 10·5, injected for 3 weeks only. Very pronounced protective stage of changes in ovary—formation of many enlarged corpora lutea. Ovaries 132 mg. Rat 220 g. $\times 18$.

FIG. 20.—Corpus luteum of normal control rat. $\times 225$.

FIG. 21.—Corpus luteum. Testosterone propionate 10·5, injected during a period of 3 weeks only. $\times 225$.

FIG. 22.—Corpus luteum. Testosterone propionate 7·5, but period of injections 53 days. $\times 225$.

FIG. 23.—Corpus luteum. Estradiol benzoate-butyrate 0·09; period of injections 53 days. $\times 225$.

As compared with the cells of the corpus luteum of a control rat (fig. 20) those of the rats injected with oestrogens or male hormones may be normal (fig. 23) or considerably hypertrophied (fig. 21). This stage of reaction is followed by the next stage, with atrophic degenerated lutein cells (fig. 22) or complete disappearance of corpora lutea.

OVARY AND CORPORA LUTEA



FIG. 18.



FIG. 19.

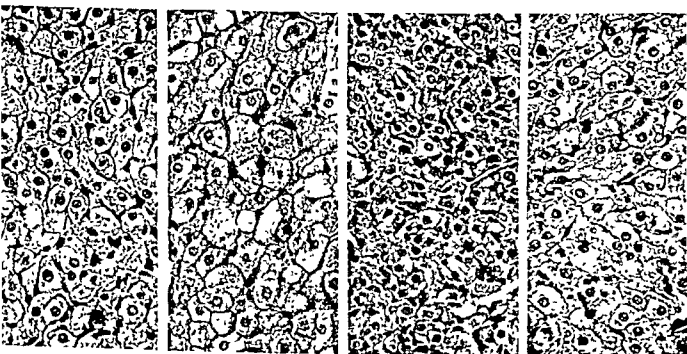


FIG. 20.

In a previous paper already mentioned (Korenchevsky, Dennison and Hall, 1937), we have described only the weights and macroscopical appearance of the ovaries of rats injected daily for 21 days with 0.5 or 1.5 mg. of testosterone propionate. A description of the histological changes is therefore given in this paper. In these short-duration experiments, both doses, especially the larger, produced in some of the rats an increased number of very large corpora lutea (fig. 19), the cells of which were hypertrophied (fig. 21). In most cases the follicles were normal and reached mature size. In only one case (fig. 18) were there more large follicles than corpora lutea, thus demonstrating a follicle-stimulating effect somewhat similar to that observed by Salmon (1938) and Starkey and Leathem (1938) in their experiments of a few days' duration. In the majority of the rats, however, the effect of testosterone propionate injected for 21 days could be described as luteinising.

Most probably a follicle-stimulating action is the first stage of the effect of testosterone on ovaries; it is usually of short duration and is followed by the second, much longer stage of pronounced luteinisation. Both these stages demonstrate a gonadotropic effect of the hormone when administered for short periods.

In our present experiments of long duration the smaller dose of the hormone produced slightly smaller ovaries than the large dose (24-30 mg., group 27 and fig. 13, compared with 33-43 mg., group 28). In the three smaller ovaries, corpora lutea were absent, or only their remnants, in the form of small patches of or scattered degenerating lutein cells, were found (fig. 13). Most of the follicles were small or atretic and only a few of medium size and normal appearance could be seen. In the largest ovary of group 28 (7.5 mg. per week) the corpora lutea, although present, were fewer than in normal ovaries and consisted of smaller and degenerating cells (fig. 22). In all the ovaries the amount of cellular interstitial tissue was increased by transformation of the cells of obliterating follicles. In some ovaries this tissue consisted of smaller cells than normal.

The prolonged administration of testosterone compounds therefore had a definite depressing effect, though varying in degree, on ovarian function and especially on luteinisation.

Simultaneous injections of oestradiol and testosterone esters (groups 29 and 30). This line of treatment was followed in most cases by the development of a gigantic uterus and vagina.

Uterus. Here the hypertrophy involved all parts of the organ. The mucosa contained patches of cellular infiltration and was often thrown into villous projections. The glands were hyperplastic and often branched, with formation of cysts. The epithelium consisted of high columnar cells usually with patches of metaplasia. The myometrium showed irregular and abnormal disposition of

the bundles in the circular layer. Thus the hypertrophy of the uterus was in several respects pathological.

Vagina. The wall of the vagina was more fibrotic than normal. The epithelial layer either consisted of hypertrophied, clear, swollen cells with dropsical vacuolation, or showed partial mucification or keratinisation. Evidently in some of the rats the mucifying effect of the testosterone predominated, in others the keratinising effect of the oestradiol.

Ovaries. In our experiments, the most striking pathological feature produced in most ovaries by simultaneous injections of testosterone and oestradiol compounds, was the formation of numerous cysts of different sizes. In three rats these cysts were very large (fig. 24), causing a tenfold increase in the weight of the affected organ. The weights of such ovaries have been excluded from table I; instead, the weight of the second ovary of the pair, *i.e.* containing no large cysts, was multiplied by 2 and the approximate weight thus obtained was tabulated.

Only the small and, rarely, the medium-sized follicles were of normal appearance. In most cases, the accumulation of follicular fluid did not proceed normally, but converted the follicles into thin-walled cysts lined with flat atrophic epithelium (figs. 25, 26 and 27). In the ovaries of one rat even the smallest follicles were very few; in two rats a few normal follicles of medium size were found. Several other follicles were in a state of atresia and transformation into interstitial tissue. The large cysts were similar in structure to those observed in some gynaecological cases, while those rat ovaries in which nearly all the follicles became moderately cystic, appeared similar to the "small cystic degeneration of ovaries" found in women (*e.g.* Miller, 1937, p. 96). Also in keeping with our results are the experiments of Steinach (1916), who observed the formation of cysts in ovaries grafted into males. A few normal corpora lutea were present in the ovaries of only one rat. In all the remaining ovaries, corpora lutea were absent (figs. 25 and 26) or small and degenerate (fig. 27), or were represented by patches of degenerate lutein cells.

The amounts of fibrous tissue and, in most cases, of interstitial tissue were abnormally increased.

Summary. 1. In the experiments of long duration, testosterone propionate injected alone in small doses into normal adult rats caused a decrease in the size and weight of the uterus, while large doses produced a gigantic uterus, abnormally distended with fluid which was either clear or contained leucocytes. The vagina was greatly hypertrophied with both sizes of dose, while in the ovaries the development of corpora lutea and follicles was arrested.

2. In the experiments of shorter duration (21 days) on the

PLATE XXXV

- FIG. 24.—Testosterone dipropionate 7.5+oestradiol benzoate butyrate 0.09. One very large cyst and, on its upper periphery, three small cysts. A few more or less normal follicles at top. On left, three cross sections of Fallopian tube. This ovary with the large cyst 287 mg., the second (atrophic) ovary 10 mg. Rat 180 g. Injections 53 days. $\times 15$.
- FIG. 25.—Testosterone propionate 7.5+oestradiol benzoate-butyrate 0.09. No corpora lutea, but several small cysts lined with flat atrophic epithelium. Stage of collapse. Ovaries 67 mg. Rat 245 g. Injections 53 days. $\times 18$.
- FIG. 26.—The same injections. Atrophic ovary with a few small cysts and a few normal follicles of small or medium size. Increased interstitial and fibrous tissues. On left centre, large follicle with a degenerated ovum, being converted into a cyst. No corpora lutea. Stage of collapse. This ovary 11 mg.; the other containing a very large cyst, 108 mg. Rat 195 g. Injections 53 days. $\times 18$.
- FIG. 27.—The same injections, but atrophic ovary contains small and degenerating corpora lutea. Small cysts. A few more or less normal follicles of small or medium size. Ovaries 29 mg. Rat 195 g. Injections 53 days. $\times 18$.

OVARY IN CROSS SECTION

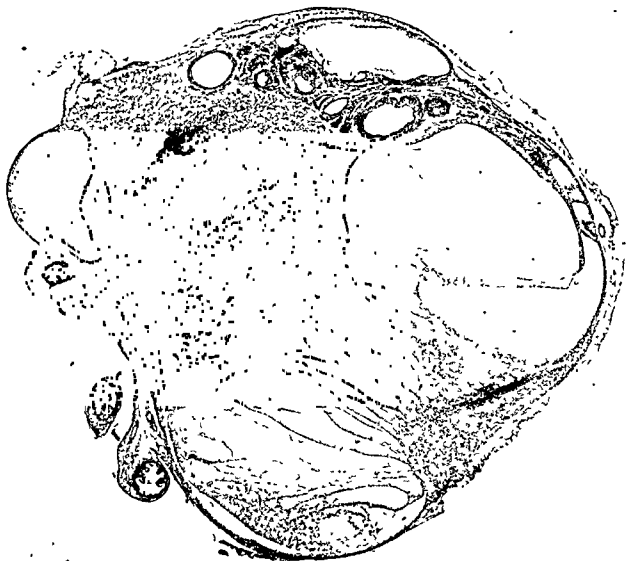


FIG. 24.

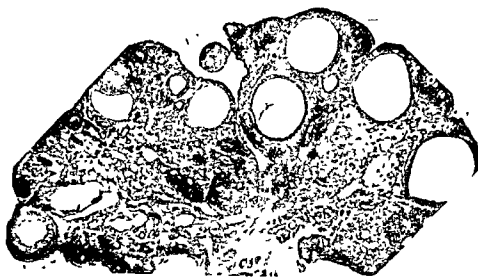
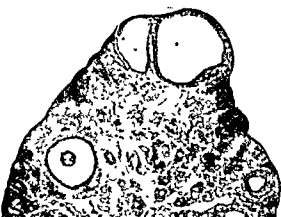


FIG. 25.



contrary a gonadotropic, chiefly luteinising effect of testosterone propionate on the ovaries was observed.

3. Simultaneous injections of testosterone and oestradiol esters were followed by the development of a gigantic uterus and vagina, the former having a pathological structure, with the development of glandular cysts. In the ovaries the follicular development was depressed, luteinisation was decreased or absent, and the formation of follicular cysts of different sizes, in some cases very large, was a prominent pathological change.

4. The results obtained by different workers, especially Mazer and Mazer, and in the previous and present papers by ourselves, indicate the possible harmful effect of testosterone propionate on the uterus and ovaries, which must be tested clinically and taken into consideration in the therapeutic application of this hormone in women.

METAPLASIA OF UTERINE EPITHELIUM AND ADENOMA-LIKE CHANGES IN THE GLANDS

Metaplasia of the uterine epithelium into squamous cells with or without keratinisation may be regarded as the first stage of the precancerous changes produced by oestrogens. It develops easily into leucoplakia (*e.g.* Hume, Burbank and Korenchevsky, 1939) which, according to several clinical observations, often becomes a "matrix" for true cancerous changes. The observations of Hinselmann (1931-32, 1932-33, 1933, 1934) are particularly convincing. Professor Hinselmann has kindly presented us with several excellent microscopical sections of the mucosa of the human cervix which strongly support this view. In them it is possible to see clearly how, in a long longitudinal section of the normal epithelial layer, metaplasia occurs in one minute area. From this slight change develops, in other cases, leucoplakia and from this in turn minute but typical cancer. The focus of early cancer may be so small that sometimes it is present in only 40 of 2800 serial sections (Hinselmann, 1934).

The literature on metaplasia produced by oestrogens and its treatment by progesterone has already been analysed (Korenchevsky and Hall, 1938), but we have been unable to obtain any references to the experimental production of adenoma in rats.

The metaplastic changes obtained in the present experiments are represented in table II, in which are summarised the data from 87 ovariectomised rats (section A) and 18 normal rats (section B). The main conclusions drawn from such a table do not differ from those drawn from the data from separate litters, since as far as possible the members of the litters have been distributed evenly among the different groups. When the number

of rats in a litter was less than the number of groups, members of litters of similar age were alternated.

Metaplasia into squamous cells of the normal columnar epithelium lining the cavity and glands of the uterus occurred with or without keratinisation of the upper rows or formation of pearls. In table II

TABLE II

Occurrence of uterine metaplasia and formation of branching glands or adenoma in the different groups of rats

	Group	Hormone and weekly dose (mg.) injected	Number of rats				
			in group	with no metaplasia in uterus	with metaplasia in uterus		with branching glands or beginning of adenoma
					slight or medium	severe or very severe	
(A) Ovariectomised rats	I	Œstradiol D or BB alone, 0.018, 0.09 or 0.2	35	12	21	2	0
	II	Œstradiol D or BB 0.018 or 0.09 + androsterone 7.5	14	1	8	5	0
	III	Œstradiol D 0.09 + dehydroandrosterone 7.5	4	2	2	0	0
	IV	Œstradiol D or BB 0.09 or 0.2 + testosterone P 2.25-7.5 or D 7.5	25	8	6	11	8
	V	Œstradiol D 0.2 + testosterone P 2.25 + progesterone 4.5	9	8	1	0	0
(B) Normal rats	VI	Œstradiol BB 0.09 alone	5	4	1	0	0
	VII	Œstradiol BB 0.09 + testosterone P or D 7.5	11	2	8	1	6
	VIII	Œstradiol BB 0.09 + androsterone 7.5	2	0	2	0	0

P = propionate ; D = dipropionate ; BB = benzoate-butyrate.

these metaplastic changes are classified for the sake of comparison into two sub-groups, the first slight (occasional metaplastic nests) or medium (involving not more than half the lumen), the second severe (affecting about three-quarters of the lumen and occasionally a few glands) or very severe (metaplasia of the whole lining epithelium of the lumen and of several glands, often with leucoplakia). The changes obtained suggested some of those, occurring in women, described and illustrated by Meyer (1930, pp. 183 and 209).

A different type of pathological change consisted in the formation of branching uterine glands (first stage), which in some cases proceeded to form adenomata (second stage). The cells lining these branching glandular or adenoma-like structures varied in size and shape and contained nuclei of varying size, often vesicular. The network of the fully developed structure (fig. 5) consisted of tubules of varying diameter, sometimes more or less distended

into cysts. The lumina were filled with secretion, either clear or containing a number of cells, chiefly leucocytes. The adenomata may undergo squamous metaplasia (fig. 6), with or without keratinisation. Meyer (1930, pp. 203, 208, 423) describes and illustrates similar changes in women.

With the doses of hormones used, the following conclusions may be suggested from the data of table II illustrated by figs. 4-8.

1. Squamous metaplasia may occur with the same frequency in ovariectomised rats receiving oestrogens alone as in those receiving also male hormones. It occurred, for example, in 66 per cent. of the rats of group I, and 68 per cent. of group IV. In group II (androsterone+oestrogen) and groups VII and VIII (normal rats injected with both hormones) the incidence appeared to be greater than in the respective controls.

2. Branching glands, the beginning of adenomata and the most severe degree of squamous metaplasia only occurred, however, in the groups injected with both male hormones and oestrogens simultaneously.

3. Even in groups II and IV, in which the most severe changes occurred frequently, some individual rats may be resistant to the metaplasia- or adenoma-producing action of the hormones. These differences in the power of resistance or perhaps in predisposition to the factors producing pathological changes might have a similar explanation to that given above in the case of other changes produced by oestradiol esters (see p. 300). These differences have been emphasised previously by Kaufmann and Steinkamm (1936), Zondek (1937a) and Korenchevsky and Hall (1938).

4. By this fact is explained our erroneous preliminary statement (Korenchevsky, Hall and Burbank, 1939) about the preventive properties of large doses of testosterone propionate. At the time, our observations had been made on only two rats which had been injected with this dose, and unfortunately both showed no metaplasia. For the same reason the conclusions suggested by the present data, apparently sufficiently numerous, may perhaps need confirmation by still further experiments.

5. The addition of progesterone prevented or much diminished the development of squamous metaplasia, which was found in only one rat of this group, and then only to a slight degree. This finding confirms the results obtained by Cesa (1936), Hisaw and Lendrum (1936) and Korenchevsky and Hall (1938) and strengthens the conclusion that progesterone in suitable doses possesses an antimetaplastic property sufficiently strong to prevent or diminish the more severe forms of squamous metaplasia. An insufficient dose of progesterone, however, has no preventive effect (Cesa, 1936; Korenchevsky and Hall, 1938; Silvestroni, 1939).

6. The absence of the most severe degree of metaplasia in

normal rats (with the doses used) as compared with that observed in ovariectomised rats may probably be explained by the fact that, in the former, corpora lutea or lutein cells have preserved their function to a greater or less degree and exert a partially protective effect. The results of Lipschütz, Murillo and Vargas (1939), who found that progesterone prevented the development of fibromyomas in guinea-pigs injected with oestradiol, support our previous conclusion that in the pathogenesis and treatment of certain tumours in human beings the sex hormones may be important factors (Korenchevsky and Hall, 1938). Burrows (1936) confirmed the antimetaplastic properties of progesterone for males in experiments on mice treated with oestrogens.

Clinical observations, however, and experiments on animals with spontaneous tumours are necessary in order (1) to investigate the possibility of a preventive or therapeutic value of progesterone for suitable tumours and (2) to determine how far the metaplasias and tumours produced by oestrogens are similar to those which develop spontaneously.

GENERAL SUMMARY

1. Experiments were performed on 181 normal or spayed female rats and various pathological changes were produced in the uterus, vagina and ovaries by different male and female sex hormones, when injected alone or in various combinations for the prolonged period of 53-146 days.

2. The co-operative activity of male hormones with oestrogens can produce in females both beneficial and pathological effects.

3. In spayed rats, amongst the beneficial effects can be included development of a larger uterus with a more normal myometrium and a larger vagina.

4. In normal rats, however, this better development may with large doses or prolonged administration of hormones, become pathological gigantism.

5. In our experiments the most important pathological co-operative effects of male hormones with oestrogens were severe metaplasia of the uterine epithelium, adenoma-like overgrowth of the uterine glands, cystic glandular hyperplasia in the uterine mucosa, and the formation of cysts, sometimes very large, in the ovaries.

6. These changes appeared to be histologically of the same kind as the similar pathological conditions which occur in women.

7. It is therefore reasonable to suggest that certain forms of these diseases in women might be caused by a disturbance in their organism of the normal balance of male and female sex hormones.

8. The first stage of the effect of male hormones on the ovaries can be defined as a follicle-stimulating action, the second stage as

a luteinising effect; this gonadotropic action then collapses and is replaced at the third stage by a toxic action on the ovaries.

9. The second (gonadotropic luteinising) and third (collapse) stages are also produced by the action of œstrogens on the ovaries.

10. Progesterone not only appears to lack any pathological action on the female organism, but in rats, if given in suitable doses, it can prevent severe metaplasia of the uterine epithelium and perhaps also some other pathological changes (adenoma, cysts).

11. Hence the rapid formation of large corpora lutea may be a neutralising autoregulation (protective stage of ovarian reaction), which protects temporarily and to a greater or lesser degree the sex organs in particular against excessive amounts of certain sex hormones. The atrophy of the ovaries appears to be the next stage (ovarian collapse) and a result of the overwhelming of this neutralising capacity by too large and toxic doses.

12. Since this autoregulation appears to be weaker in some animals than in others, the ovarian collapse in such animals might occur rapidly, which probably explains the apparently conflicting final results of the experiments. The stage of collapse might be complicated by the development of cysts.

13. In general a variable response of individual animals to the action of the injected sex hormones seems to be related to individual variations in functional capacity and inter-relation of the endocrine organs in different animals.

14. Since metaplasia of the uterine epithelium may be considered as the first stage of the precancerous changes produced by œstrogens, the same disturbance of the ratio of male and female sex hormones may perhaps play a part in the development of some human tumours, for treatment of which the effect of progesterone should be investigated.

Grants from the Medical Research Council, The Lister Institute and the Ella Sachs Plotz Foundation have enabled us to carry out this work and to them our thanks are due. We are much indebted to Miss M. A. Ross who prepared the photomicrographs, to Messrs Organon Ltd. for the supply of progesterone and to Messrs Ciba Ltd., in particular to Dr K. Miescher, for all the other hormones.

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576.851.5 (*Hæmophilus influenzae*) 616—022.6 :
616.233—002

HÆMOPHILUS INFLUENZÆ AND INFLUENZA VIRUS IN RELATION TO BRONCHITIS*

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MOST authors mention *Hæmophilus influenzae* as a cause of bronchitis and bronchiolitis during influenza periods, although possibly as a secondary invader. Since the discovery of the virus of influenza the question has more than once been raised of the significance of the *Hæmophilus* group in human pathology, and one still meets the idea that in influenza in particular it may be a cause of secondary bronchial and pulmonary complications (Beclere, 1937, 1938, Schmidt and Kairies, 1938). There are a few publications prior to 1933 (Sacquépée, 1901, Bezançon and de Jong, 1905, Davis, 1908, Luetscher, 1915, Hammond *et al*, 1917, Abrahams *et al*, 1917, Rosenthal, 1923) and others since (Fothergill and Chandler, 1936, Mulder, 1938) in which attention is drawn to the occurrence of *H. influenzae* in the purulent sputum of ordinary "primary" bronchitis, with no epidemiological relationship to epidemic or pandemic influenza. On the other hand one often finds mention of it in bronchiectasis or in chronic purulent bronchitis (Boggs, 1905, Vogt, 1911, Bruckner *et al*, 1912, Bossert and Leichtentritt, 1921, Elkeles, 1931, 1937). The older investigators, contemporaries of R Pfeiffer, sometimes spoke in such cases of chronic influenza. The article of Luetscher on this subject is well worth reading.

It is difficult to estimate the value of reported investigations of the significance of this group in the pathology of the air passages, since the mere presence of these bacteria has often been regarded as equivalent to the clinical diagnosis of influenza. It may further be assumed that a general search for *H. influenzae* only began when influenza prevailed in pandemic form in 1918. The bacteriology of common purulent bronchitis has been but little studied in clinics. That of non foetid chronic purulent bronchitis on the other hand is better known, presumably because such cases are often submitted to observation for differential diagnosis from tuberculosis. The secondary bronchopneumonia in measles and whooping cough has however often been studied bacteriologically (Jochmann, 1909). Recently (Pittman, 1931, Fothergill and Chandler, 1936) it has

* The virus investigations were made possible through the assistance of the Jan Dekker Fund.

been supposed that the respiratory strains of *H. influenzae* may be non-pathogenic R forms of the smooth strains of Pittman. That these smooth strains should be the cause of the purulent bronchitis and bronchiolitis in influenza is improbable for the reason that in inter-epidemic periods they are continuously present in the community and subtype b apparently gives rise only to general infections such as meningitis and sepsis in infants and young children (Cohen, 1909; Kapsenberg, 1929; Neal *et al.*, 1934; van Lookeren Campagne, 1937-38; Mulder, 1937, 1939). We have occasionally found this type in smooth form in purulent sputa from adults suffering from mild, partly post-operative bronchopneumonia, bronchitis and pneumococcal pneumonia, completely independently of an influenza epidemic and without a clinical picture of influenzal pneumonia.

It is difficult to assess the pathogenic significance of strains of the respiratory type cultivated from purulent sputa. Blake and Cecil (1920) with one strain were able to produce in monkeys purulent sinusitis, bronchitis and bronchopneumonia and even sepsis with purulent pericarditis. It is not quite certain, however, whether the strain was of the respiratory type, as it had been isolated from pus from an empyema in a child suffering from pneumonia and it is particularly in these empyemas that strains of type b can be found (Pittman; van Lookeren Campagne). Tests have shown only very slight susceptibility of human adults to the respiratory type. Only occasionally has it been possible to produce infection and then only a mild tracheitis or purulent naso-pharyngitis (Cecil and Steffen, 1921; Park and Cooper, 1921; Fenyvessy and Kopp, 1926; Walker, 1928; Smorodintseff *et al.*, 1936). The best proof at present of their pathogenic properties is the fact that they can often be found in pure culture in cases of purulent mastoiditis and that we have isolated them from all our cases of purulent capillary bronchitis and from one case of purulent meningitis (Mulder, 1939). Nothing is known of the possibility of the existence of sub-types varying in pathogenic properties. Further investigation on this line is desirable.

Influenza and primary acute purulent bronchitis (bronchiolitis)

Although no large series of cases of primary acute bronchitis and bronchiolitis has been examined for the presence of an influenza virus it is quite unjustifiable on clinical grounds to group these conditions with the influenza-like diseases.

Epidemics of purulent catarrhal affections do not show such an acute course as those of influenza. In the Tropics one of the periods of catarrhal affections observed by us lasted 4 months. In Europe a seasonal incidence exists for these diseases, chiefly in winter and lasting till far into spring. Epidemic influenza on

the other hand arises there every second to fourth year in sudden explosions, usually during December, January or February.

In our experience an epidemic of influenza includes a relatively large number of patients without any complicating purulent catarrhal process in the trachea or bronchi. During the influenza epidemic of February 1939 in Groningen we saw about 80 cases, almost all of whom were locally garrisoned soldiers. The diagnosis of influenza was substantiated by the demonstration of influenza virus in ferrets and in mice. Two cases only had purulent catarrh of the air passages, one associated with *Str. hæmolyticus* (type 5), the other with *H. influenzae* of the respiratory type in the sputum. Otitis and bronchopneumonia were rare complications and were exclusively caused by hæmolytic streptococci, all type 5.

Purulent catarrh of the air passages is also seen apart from the seasonal disease. In Holland we have seen such cases in May, June and July, completely independent of any prevailing influenza or influenza-like disease. Two isolated cases occurred, one in a garrison (see below), the second in a household consisting of husband, wife and three children. This slight contagiousness is vastly different from that of epidemic influenza.

An important clinical difference from influenza is the fact that, in purulent catarrh the exudation of muco-pus always begins early (first or second day of disease).

In our experience secondary infection of the lung alveoli with pyogenic cocci is very rare in purulent catarrh as compared with influenza. In the accompanying table are given our findings regarding the incidence of *H. influenzae* in acute and chronic purulent

TABLE

Incidence of H. influenzae in acute and chronic non-fetid purulent catarrh of the air passages and in bronchopneumonia (1928-1939)

Disease	Total cases	Probably secondary to Influenza	<i>H. influenzae</i> found (per cent.)	<i>H. influenzae</i> the predominating organism (per cent.)
Acute purulent broncho-bronchiolitis	25	3	100	75
Acute purulent bronchitis . . .	120	25	90	43
Acute purulent tracheitis . . .	78	11	97	36
Chronic purulent bronchitis (non-fetid)	59	0	95	60
Acute bronchopneumonia . . .	20	0	85	Associated with cocci
Post-operative lung conditions .	14	0	99	Associated with cocci
Secondary bronchitis and broncho-pneumonia	12	0	75	0
Pulmonary tuberculosis . . .	19	0	36	0
Nose and throat passages . . .	54	0	50	0

Of 97 strains examined for growth factor requirements 90 required both X and V factor, 1 V factor only, while 6 grew on Levinthal agar and blood agar only.

catarrhal affections of the air-passages occurring during 1928-1939. A small proportion of the acute cases were certainly secondary to influenza.

To what extent still unknown viruses are responsible for acute purulent bronchitis is unknown, but although further investigation in this line is necessary we do not consider this probable on clinical or epidemiological grounds. We report a case observed in the summer of 1938 in the garrison at Groningen in which the absence of the influenza virus was as good as proved. It arose as an isolated case independent of epidemic influenza.

Case report. H. M., age 20 years. Previously always well but had acute bronchitis three years ago.

22.7: felt ill with a "nasal cold" (?) and a general feeling of malaise. 23.7: reported ill but did not show fever. In the afternoon he began to cough. 24.7: feverish. 25.7: temperature did not rise above 37.8° C. 26.7: temperature rose suddenly to 40.2° C. On examination patient did not appear very ill. No nasal discharge, conjunctivitis or tonsillitis. Fauces slightly injected. In both lungs numerous moist râles at bases; no dullness on percussion and no bronchial breathing. Purulent sputum. Blood culture negative. Leucocyte count 19,700 per c.mm.; 10 eosinophils per c.mm. Sedimentation rate 27 mm. per hour. X-ray plate of thorax showed no pulmonary foci.

The course of the disease was uneventful. 17.8: moist râles still audible in left lower lobe. Expectoration ceased.

Sputum examination. No tubercle bacilli on cultivation. Gram's stain showed innumerable cocco-bacilli similar to *H. influenzae*. Culture on Levinthal agar yielded *H. influenzae* of the respiratory type (no light refraction; growth only in presence of X and V factors; no hæmolysis; indole formation strongly positive). On the first blood agar plate three colonies of pneumococci, type undetermined. No other organisms present. Of 4 mice injected intraperitoneally with sputum, only one died after seven days of sepsis due to a pneumococcus of undetermined type. Repeated cultivation of the sputum gave the same result. The pneumococci remained scarce and were found with difficulty. On 6.8 microorganisms were no longer seen in films. The patient's serum 18 days after admission did not agglutinate the *Hæmophilus* strain. One ferret was instilled intranasally under light ether anaesthesia with an emulsion of sputum of 26.7 and 27.7 and a second with the garglings of 27.7. Neither showed catarrh or fever. The serum of the first ferret after 20 days showed no protective antibodies against influenza virus, strain WS, received a year before through the kindness of Dr Wilson Smith. Both ferrets were reinfected after 40 days with the WS strain and both developed symptoms of influenza.

The serum of the patient 18 days after admission showed no rise in antibody content against the virus strains WS and P.R.8 as compared with the serum of the first day of high fever (26.7). Strain P.R.8 was used at the suggestion of Dr Wilson Smith, because of its polyvalency (Smith and Andrewes, 1938) and because we did not then possess a strain isolated in Holland.

From this case it appears that *H. influenzae* can be found in the purulent sputum of cases of purulent bronchitis in the absence of influenza virus.

Considering all the evidence we think it probable that organisms of this group are a common cause of primary and secondary acute and chronic purulent inflammation of the bronchi and bronchioles. The future will show to what extent still unknown viruses are of primary significance in ordinary bronchitis.

Summary

1. *H. influenzae* of the respiratory type is found independently of any prevalent epidemic or pandemic of influenza, in the majority of cases of ordinary acute and of non-fœtid chronic purulent bronchitis (bronchiectasis), often in predominating numbers and especially in cases of pure capillary bronchitis.

2. The pathogenic properties of these organisms have not been definitely proved but it appears highly probable that they can cause acute and chronic purulent inflammation in the bronchial mucosa.

3. It appears improbable that most acute cases are caused primarily by viruses and that *Hæmophilus* infection is only of secondary importance.

4. A description is given of a sporadic case of acute purulent bronchitis in which *H. influenzae* of the respiratory type was found in almost pure culture and in which attempts to demonstrate the presence of influenza virus were completely unsuccessful.

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OBSERVATIONS ON POLYARTHRITIS AND ON EXPERIMENTAL *ERYSIPELOTHRIX* INFEC TION OF SWINE

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(PLATES XXXVI XLI)

EARLY bacteriological investigations of arthritis in swine undertaken by Glasser in 1912 and by Stutzel in 1913 are described by Schermer and Ehrlich (1922). Glasser attributed the arthritis, associated in his cases with fibrinous inflammation of pleurae, pericardium and peritoneum, to bacteria which he named "Serosenbazillen". Stutzel was unable to find any bacteria in similarly diseased animals and attributed the arthritis to injury during transport. Schermer and Ehrlich confirmed Glasser's findings and stated that the "Serosenbazillus" morphologically resembled the bacillus of swine erysipelas but was Gram negative. Meyerhofer (1935) found a similar organism in the joints, blood and liver in 9 out of 27 young arthritic pigs.

Various authors have described suppurative arthritis of swine due to *Corynebacterium pyogenes*, but the lesions (Ward, 1917) differ in many important respects from those of the disease with which we are now concerned. Sekiguchi and Irons (1917) found streptococci in the joints of some cases of chronic suppurative arthritis. In other cases in which there was proliferation of the synovial tissues, with or without cartilage destruction but with no gross evidence of suppuration, they isolated a Gram positive bacillus which differed from *C. pyogenes* in its failure to liquefy gelatin and coagulated serum, with it they were able to reproduce the disease in swine. From pigs' legs exhibiting various stages of arthritis Ward (1922) in many instances isolated *Erysipelothrix rhusiopathiae*, which he concluded was the same organism as that found by Sekiguchi and Irons. He recovered the bacillus from the majority of joints in the early stages of the disease, but those showing advanced lesions with permanent bony enlargement were usually sterile. He also produced arthritis in a pig by intravenous injection of cultures. He recognised the possible fallacies of drawing conclusions from abattoir material and stressed the desirability of field observations. McGinnis and Spindle (1934) isolated *E. rhusiopathiae* from bone material and bone waste from the slaughter house at a bone button factory. There is no doubt

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that the organism leads a saprophytic existence in animal material and may easily contaminate cultures made in such surroundings as the abattoir.

Although *E. rhusiopathiae* had been recognised as the causative organism of swine erysipelas in Europe since its discovery by Pasteur and Thuillier (1882) and in America since 1921 (Creech, 1921-22), the recognition of arthritis as a chronic form of this infection came slowly. Parker, Lockhart and Ray (1923-24), in post-mortem work at slaughter-houses, noticed the association of polyarthritis with diamond skin disease and with necrotic dermatitis, and *E. rhusiopathiae* was the only suspicious organism they were able to isolate from skin, joints or other tissues. Craig (1926) in Dublin was one of the first in the British Isles to describe polyarthritis as a chronic form of swine erysipelas, which he stated may arise independently or may follow any of the more acute manifestations. It occurs chiefly in the winter months and usually after weaning, though cases are not unknown in sucking pigs. He failed to obtain organisms from the joints, bone marrow, blood or spleen. One case occurred in association with verrucose endocarditis. Creech (1933) successfully infected 11 out of 22 pigs with cultures of *E. rhusiopathiae*; 4 developed acute arthritis after intravenous inoculation and one died from acute swine erysipelas after infection by feeding.

Ducksbury (1934) in England drew attention to enlargement of the iliac and inferior cervical lymph glands as an indication of the presence of arthritis in the limbs, and by this means identified 30 cases of arthritis in 22,000 slaughtered bacon pigs. *E. rhusiopathiae* was grown from 4 out of 6 of these limbs and a diphtheroid organism from one other. Ducksbury's figures, however, do not give a true idea of the economic importance of arthritis in swine, as a great majority of affected animals are killed long before they are old enough to be sold for bacon. Buitenhuis (1935) demonstrated *E. rhusiopathiae* in the affected joints of 26 out of 30 cases (87 per cent.) of swine arthritis with severe exudative and proliferative inflammation of the joints and enlargement of the deep inguinal glands. Where the joints were sterile, the arthritis was in a more chronic stage. He found lesions and organisms in the joints of the lumbar vertebrae in many cases. Wernery (1937) made careful observations on the pathology of swine erysipelas arthritis which he described as "arthritis chronica villosa" as distinct from "arthritis chronica deformans." He recovered the bacillus from 12 out of 20 joints examined and noted that the joint disease usually occurred without any other manifestation of swine erysipelas.

Kernkamp (1937) and Schoening (1938) have both suggested that swine arthritis may be due to infection with more than one type of organism. Kernkamp named many organisms found in different types of arthritis, the most important being *E. rhusiopathiae*, *C. pyogenes* and *Br. abortus*.

Creech (1937), like Craig (1926) and Hutyrá, Marek and Manninger (1938), asserts that polyarthritis is a frequent form of chronic swine erysipelas and that arthritic animals may never have shown evidence of the acute or subacute disease. Pullar (1938) observed chronic arthritis in 2 animals in one of the first outbreaks of swine erysipelas recorded in Australia.

E. rhusiopathiae is known also to cause arthritis in sheep (Poels, 1913; Cornell and Glover, 1925; Hopkirk and Gill, 1930; Marsh, 1933; Murnane, 1938), affecting principally lambs, and it is thought that infection commonly takes place by way of the umbilicus or open docking or castration wounds. The disease has been artificially induced in lambs as well as in pigs. Sero-fibrinous arthritis due to *E. rhusiopathiae* has also been observed in horses injected with living organisms for the production of antiserum (Goerttler, 1926) and some tentative experiments have been made on the localisation of this organism in the joints of rabbits (Goret and Jean, 1934).

The observations and experiments now to be reported include bacteriological and pathological observations on 9 animals suffering from naturally acquired polyarthritis, and on 23 others which have been the subject of experiment. For identification all animals received a letter with or without a number. Animals with the same letter (D 1, D 2) were litter-mates; those with different letters came from different stock and different farms except pigs M, Ma 1 and Ma 2, which came from two litters on the same farm.

Joints will be referred to not by the nomenclature used by farmers and veterinary surgeons (*e.g.* hock, stifle), as these terms may lead to confusion, but either by their anatomical description (*e.g.* radio-carpal) or by the names of their analogues in man (*e.g.* knee, wrist, ankle).

Technique

Care and feeding of animals. All pigs were of large or middle white breed and were reared in Yorkshire. Those used for experiment were purchased soon after weaning and kept in large loose boxes on the experimental farm of the Department of Experimental Pathology and Cancer Research, University of Leeds. The pens were stone floored and adequately bedded with straw. Not more than 4 animals were housed in each. Diet consisted of balanced rations of fish meal (2 cwt.), sharps (11 cwt.), barley meal (2 cwt.), Uveco (3 cwt.), and minerals (2 stones) unless otherwise stated, and was given in adequate quantity to maintain health but not to force fattening. Kale was added to the rations of some animals. Care was taken that badly crippled animals were able to reach their food. Certain animals died of disease; others were killed either by shooting or by chloroforming.

Pathological. Post-mortem examinations were mainly conducted in the farm laboratory or out-of-doors and included naked eye examination of the organs and the taking of material for bacteriological and histological examination from most of the thoracic and abdominal viscera. The gastro-intestinal tract was seldom opened and examined throughout its full length unless a survey of its serous aspect suggested some pathological condition. All the larger joints were dissected and examined after material had been removed for bacteriological purposes. X-ray examinations were occasionally made of limbs or of the spine removed *en bloc*. Soft tissues of the joints and pieces of viscera were embedded in paraffin, and celloidin sections of bone ends were made after decalcification. Extensive histological examination was made of almost every joint which showed naked eye evidence of arthritis. Sections were stained routinely by hæmatoxylin and eosin or van Gieson. Other stains used were Gram, pyronin-methyl-green, Cajal's fibrous tissue stain, elastic tissue stain, Foot-Wilder's reticulum stain, phosphotungstic acid hæmatoxylin and Turnbull-Jenner.

Bacteriological. For serological tests and for culture blood was collected during life from an ear vein after suitable preparation of the skin and from the heart *post mortem* after searing the pericardium. Cultures from other viscera were made *post mortem* in a similar manner. Cultures from the joints were usually made in the laboratory from disarticulated limbs; the skin, superficial muscles and fasciæ were dissected off the unopened joint, the capsule seared and material aspirated by means of a Pasteur pipette. An attempt was always made to withdraw some synovial tissue as well as fluid.

Culture media. The following media were employed.

- (a) *Heated blood broth* containing 10 per cent. ox blood, heated for 10 mins. at 75° C.
- (b) *Buffered meat broth* prepared by extraction at low temperature; a modification of the medium described by Todd and Hewitt (1932).
- (c) *Serum bouillon* containing 10 per cent. sheep serum.
- (d) *Fresh blood agar* containing 12 per cent. rabbit blood.
- (e) *Heated blood agar.* Medium (d) heated at 75° C. for 10 mins.

Heated blood broth proved the most generally useful medium for the recovery of organisms from blood and joints.

Agglutination of E. rhusiopathiæ. Three strains of *E. rhusiopathiæ* were grown on heated blood agar for 24 hours at 37° C., suspended in 1 per cent. carbol-saline and left in the ice chest for 48 hours. They were strain 1224 of the National Collection of Type Cultures, and strains A and B, isolated from pigs A and B (see p. 332). Of the bacterial suspension 0.5 c.c. was mixed with 0.5 c.c. of varying dilutions of pig's serum. Readings were taken after 6 hours' incubation at 37° C. and again after 24 hours. Latterly a somewhat different technique was used. The antigen consisted of 18-hour broth cultures and readings were made after 2 hours in a water-bath at 55° C. and again after standing overnight in the ice-box. Comparable results were obtained on the same sera by both methods, but the end point was clearer with the second technique. A control high titre serum was put up with each day's tests.

Titration of streptococcal antihæmolysin. Hæmolytic streptococci from a strain supplied by Professor J. W. McLeod were grown for 18 hours in the medium described by Abdalla and McLeod (1939). The culture was then passed through a Masson filter and the first 25 c.c. of filtrate discarded. The remainder was used for titration. The minimal hæmolytic dose (M.H.D.) was estimated as the minimum amount required to hæmolyse completely 0.5 c.c. of 5 per cent. washed ox red cells. Dilutions of serum ranging from 1:10 to 1:640 were then put up with 0.5 c.c. of a dilution of hæmolysin containing 2.5 M.H.D., and 0.5 c.c. of 5 per cent. washed ox red cells added. The tubes were incubated at 37° C. for 2 hours and the end point was taken to be the last tube showing no hæmolysis. The antihæmolysin content was expressed as the number of M.H.D. neutralised by 1 c.c. of serum. For each day's titration a fresh hæmolysin was prepared.

NATURALLY ACQUIRED ARTHRITIS

Seven pigs suffering from arthritis were collected from various farms in North and East Yorkshire and observed under laboratory conditions for varying periods before death. Two others acquired arthritis naturally while under observation. Brief protocols of these nine animals follow.

Fig M. Suckling gilt, 8 weeks old. Symptoms of arthritis for 3 weeks. **Post-mortem examination.** Mild inflammatory polyarthritis. Clear pericardial effusion; hæmorrhage into apical muscle of left ventricle with microscopic evidence of degeneration of adjacent myocardium; enlargement of mesenteric lymph nodes. No bacteriological evidence of aetiology.

Fig Ma1. Hog, 16 weeks old. Arthritis for 6 weeks since weaning. Failure to fatten. Rectal temperatures up to 105° F. Other litter mates

also showed some rheumatism but were subsequently marketable. Arthritis of wrists, knees and ankles which were red, swollen and painful. **Post-mortem examination.** Mild inflammatory polyarthritis. Clear pericardial effusion; organising bronchopneumonia and pleurisy; mesenteric lymph node enlargement. No bacteriological evidence of aetiology.

Pig Ma 2. Hog, 16 weeks old. Condition similar to Ma 1. **Post-mortem examination.** Mild inflammatory polyarthritis of unknown aetiology. Clear pericardial effusion; bronchopneumonia; hyperplasia of mesenteric glands.

Pig A. Gilt, 17 weeks old. Arthritis for 8 weeks. Severely crippled and lean (fig. 1a). The only arthritic animal in a large herd. **Post-mortem examination. Joints.** The main joints of all four limbs were affected by severe arthritis, with considerable synovial proliferation and slight excess of moderately turbid joint fluid. Adherent pannus crossed the cartilages of the femur (fig. 2) and there was superficial erosion of articular cartilage in this and other joints, but no destruction of bone was revealed by X-ray examination (fig. 3). Microscopic examination of joint tissues showed inflammatory changes—increased vascularity, proliferation of synovial lining cells, lymphocytic and plasma cell infiltration, hyalinisation of some synovial villi and vascular granulation tissue (pannus) adherent to articular cartilage (fig. 4). Gram-positive bacilli were seen in sections and appeared to lie among the cells and fibrin deposit on the surface of the synovial membrane (fig. 5). Cultures from the knee were sterile. **Other organs.** Bilateral empyema and mediastinitis due to combined infection with *Str. haemolyticus* and *E. rhusiopathiae* (fig. 6). Suppurative pericarditis; focal necrosis of myocardium with little cellular reaction (fig. 7). One week before death, serum agglutinated *E. rhusiopathiae* to 1:320.

Pig B. Hog, 52 weeks old. Arthritis for 38 weeks. Clinical ankylosis of many joints (fig. 1b) with high arched back, but no spinal lesion was detected on X-ray examination of the spine removed *post mortem*. **Post-mortem examination. Joints.** Chronic proliferative polyarthritis of a non-suppurative type affecting all the joints of all four limbs except those of the digits and the left elbow joint. *E. rhusiopathiae* was isolated from one affected joint; two others were sterile. Bony deformity due to overgrowth of epiphyseal bone (fig. 8) caused enlargement of the joints during life. There was no healthy synovial lining in any of the diseased joints. Lymphoid cell infiltration of the synovial tissues was prominent microscopically (fig. 9). Plasma cells were also present but polymorphs and eosinophils were rarely seen. Fibrous adhesions extended across many of the joints and chondrification and ossification of adherent inflammatory fibrous tissue was observed on the head of the femur in the left hip joint, which showed firm fibrous ankylosis (fig. 10). **Other organs.** Enlargement of mesenteric and iliac lymph nodes; gas gangrene infection (possibly P.M.) of one iliac gland; localised subcutaneous abscess of cheek yielding pure growth of *Str. haemolyticus* group C. Serum agglutination titre against *E. rhusiopathiae* was 1:40, 1:160, 1:320 and 1:25 at successive monthly examinations. No agglutination of *Br. abortus*, *melitensis* or *suis*.

Pig C. Hog, 36 weeks old. Arthritis for 13 weeks, becoming progressively more crippling. Death four days after chloroform anaesthesia for exploration of joint. The extensive central necrosis of the liver lobules suggested delayed chloroform poisoning. **Post-mortem examination.** Gross arthritis in elbow, knee and subastragaloid joints, slight in shoulder, hip and ankle. Microscopic examination of joint tissues showed congestion, fibrosis and proliferation of synovial lining cells and lymphocytic infiltration around the deeper blood vessels (fig. 11). Cultures from two joints were

negative. There was trichinosis of the myocardium and heavy nematode infestation of the bronchi.

Serum agglutinated *E. rhusiopathiae* at 1 : 40 and 1 : 160 on two examinations. No agglutination of *Br. abortus*, *suis* or *melitensis*.

Fig E. Hog, 19 weeks old. Arthritis for 7 weeks. Very emaciated and much crippled. **Post-mortem examination.** **Joints.** Chronic proliferative polyarthritis. Foci of fibrinoid necrosis were observed microscopically in the deeper synovial tissues of affected joints (figs. 12 and 13), which with their surrounding cellular reaction of fibroblasts and histiocytes strongly resembled the early changes in the subcutaneous nodules of rheumatoid arthritis of man (Collins, 1937). Polymorphs, though present in fair numbers in smears of the joint effusion, were rarely seen in the synovial tissues. **Other organs.** Early pneumococcal empyema. Recent subcutaneous abscess of abdominal wall containing pneumococci. Blood culture *post mortem* yielded a pure growth of group IV pneumococci. Two weeks before death serum agglutinated *E. rhusiopathiae* to 1 : 80; no agglutination with *Br. abortus*, *melitensis* and *suis*.

Craig produced some evidence that the arthritic form of swine erysipelas was contagious. Young pigs placed in contact with arthritic animals developed a mild form of arthritis after an interval of about 3 months. Hutyrá, Marek and Manninger state that natural infection with swine erysipelas takes place from food contaminated by excreta, from soil infection, and possibly from carriers. General observations, however, on large herds in North Yorkshire show that usually only a few animals are affected with arthritis. This polyarthritis, therefore, does not possess an epizootic character. In order to enquire into the matter of contagion, we experimented with four healthy pigs of the same litter, 8 weeks old and recently weaned (D 1-4). D 2 and D 3 were placed in the loose box occupied by arthritic pigs B and C. D 1 and D 4 served as controls and were kept in another part of the farm far removed from the arthritic stock. D 2 developed arthritis within 4 days and D 3 within 10 days of being placed in contact with the arthritic animals. The control pigs remained healthy and agile and were subsequently used for other experiments.

At the time of this experiment we were not sure of the aetiology of the arthritis in pigs B and C. Subsequently, swine erysipelas infection was proved in pig B and was thought to be the probable cause of the arthritis in pig C. It is particularly interesting that pigs D 1 and D 2 developed arthritis but at no time showed skin manifestations of swine erysipelas. Protocols of these two animals follow.

Fig D 2. Gilt, 21 weeks old at death (28.5.38). Duration of arthritis 12 weeks. **History.** 26.2.38. 8 weeks old. 1.3.38. Healthy and active; placed in loose box with D 3 and arthritic animals B and C. 5.3.38. Acute arthritis of forelegs (fig. 1c). 14.3.38. Right wrist exposed under chloroform. Fair quantity of slightly turbid joint fluid aspirated. Aerobic cultures of fluid and blood sterile. 29.3.38. Serum agglutinin titre for *E. rhusiopathiae* = 1 : 160. 7.4.38. Clinical arthritis of wrists and knees.

Animal lean and ill On account of poor condition removed from contact with arthritic stock 14 4 38 The animal had been run out on grass daily and became much more agile Some cough Striking difference in size between this pig and healthy litter mates 21 4 38 Agglutination titre against *E. rhusiopathiae* (strains A and 1224) = 1 160 No agglutination of *Br. suis*, *abortus* or *melitensis* 27 5 38 After a month of general improvement in health animal suffered a relapse and movements again difficult It died during the following night **Post-mortem examination** 28 5 38 **Nutrition** Very ill developed animal Body weight 47 lb (healthy litter mate D 4 111 lb) **Joints** Right and left hip, radio carpal, elbow and shoulder joints affected No naked eye evidence now of arthritis in knees The most affected joints showed a ring of injected enlarged synovial villi around the margins of the articular surfaces In left shoulder great excess of slightly turbid, blood stained fluid, sheath of biceps tendon distended with effusion and communicating with joint sac Both tendon sheath and synovial lining covered with proliferated and injected synovial villi Head of left humerus flattened owing to collapse of epiphysis Ulceration of cartilage not observed in any joint and synovial pannus nowhere extended over cartilage more than a few mm from the margin Microscopical appearances those of non suppurative proliferative arthritis Smears of effusion in left shoulder showed majority of cells to be mononuclear forms Few polymorphs and no organisms Cultures from left shoulder *post mortem* yielded only *Staph. albus* probably a contaminant **Other organs** Hypostatic pneumonia

Fig D 3 Hog 17 weeks old at death (2 5 38) **History** 27 2 38 8 weeks old 1 3 38 Together with D 2, brought to live in contact with arthritic animals B and C 10 3 38 Clinical evidence of arthritis of joints of left hind leg 18 3 38 Arthritis increasing Left ankle joint exposed and synovial fluid aspirated under chloroform Cultures of this and of blood sterile 14 4 38 Arthritis still present but not progressing severely Animal small and ill developed 2 5 38 Found dead **Post-mortem examination** **Joints** Arthritis present in both knees, absent in both elbows, other joints not examined Small inflamed synovial villi distributed throughout left knee joint Moderate pannus formation **Other organs** Hemorrhagic infarction of lungs Focal myocardial degeneration with commencing fibroblastic reaction Gas gangrene of liver so extensive as to suggest ante mortem infection Agglutination titre of serum against *E. rhusiopathiae* did not exceed 1 40 3 weeks after onset of arthritis No agglutination with *Br. abortus*, *melitensis* or *suis*

These nine animals comprise the material upon which our observations of the natural arthritis have been made Direct evidence of the aetiology was unobtainable in some animals and was not very definite in others Certain pathological features, however, common to all animals led us to regard all the cases as falling within a single pathological though not necessarily a single aetiological group The condition of the joints in general may be described as a polyarticular non suppurative proliferative arthritis of subacute or chronic form The animals also tended to display the same associated pathological lesions—pericardial effusion pleurisy and lymphadenopathy (table I) Joints and blood were sterile, except in two instances which will be discussed The

progressive history, the failure to fatten and the stunted growth, as well as other clinical appearances, were more or less alike in all cases.

Table I summarises the pathological characteristics of the arthritis. Proliferation of the synovial villi as well as of the lining

TABLE I

Summary of pathological findings in joints and other organs in naturally acquired polyarthritis of swine

Animal	M	Ma 1	Ma 2	D 3	E	A	D 2	C	B
Duration of arthritis (weeks)	3	6	6	7	7	8	12	13	38
Gross appearances of the joints									
Effusion	±	—	±	—	++	+	+	—	—
Villous overgrowth	±	±	±	+	+	++	+	+	++
Pannus	—	—	—	+	+	+	±	+	+
Fibrous ankylosis	—	—	—	—	—	—	—	—	—
Cartilage erosion	—	—	—	—	+	+	—	+	+
Deformity of epiphyseal bone	—	—	—	—	—	—	+	—	+
Microscopical appearances of the joint tissues									
Hyperæmia	+	+	+	+	+	+	+	+	+
Proliferation of synovial lining	±	+	+	+	+	+	+	+	+
Lymphoid cell infiltration	±	±	±	±	+	+	±	+	++
Focal collections of lymphocytes	—	—	—	—	—	—	—	+	+
Focal collections of polymorphs	—	—	—	—	+	—	—	—	—
Eosinophil infiltration	—	—	—	—	—	+	—	+	±
Connective tissue degeneration	—	—	—	—	+	—	—	+	—
Fibrosis	—	—	—	—	+	+	+	+	++
Hyalinisation of villi	—	—	—	—	—	+	—	+	—
Cellular reaction in sub-articular bone	—	—	—	—	—	+	—	+	+
Lesions in other organs									
Lungs and pleuræ	—	+	+	+	+	+	+	—	—
Myocardium	+	—	—	+	—	—	—	—	—
Pericardial effusion	+	+	+	+	—	+	+	—	+
Mesenteric lymph nodes	+	+	+	—	—	—	—	—	—
Serological									
Highest agglutination titre with <i>E. rhusiopathiæ</i>	1:40	1:80	1:320	1:160	1:160	1:320
Highest streptococcal anti-hæmolysin titre	5120	200	480	6400	960	1000

PLATE XXXVI

FIG. 1.—Arthritic swine.

- (a) *Pig A*. Natural arthritis of 8 weeks' duration.
- (b) *Pig B*. Natural arthritis of 20 weeks' duration.
- (c) *Pig D 2*. Acute arthritis, 2 days, after four days' cohabitation with arthritic swine.
- (d) *Pig G 2*. Experimental arthritis of 9 weeks' duration following intravenous injections of *Erysipelothrix*.

The characteristic flexion deformity of the forelegs is due to inability to carry weight on the extended carpus and disappears on lying down. It implies painful arthritis of elbow or carpal joints. The arched back and adducted and ankylosed hips of pig B are common in the more chronic cases. Swollen joints are seen in all.

FIG. 2.—*Pig A*. Arthritic knee joint showing extensive proliferation of synovial tissues partially adherent to articular surfaces of femoral condyles and encroaching on articular margins of patella (reflected upwards). Specimen on left is the knee of pig L 1 and shows no arthritic change.

FIG. 3.—*Pig A*. X-ray photograph of grossly arthritic knee (see fig. 2). No bony changes as seen in suppurative arthritis. $\times 1$.

POLYARTHRITIS OF SWINE



FIG. 1 a.



FIG. 1 b.

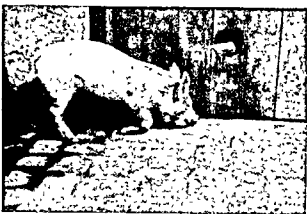


FIG. 1 c.



FIG. 1 d.



FIG. 2.



FIG. 3.

layer of synovial cells and hyperemia of the synovial membrane were observed in all affected joints. In mildly affected joints these changes were confined to discrete areas of the synovial membrane. The commonest site for commencement of the proliferative changes was at the level of the inter joint line around the articular margins. In severely affected joints the overgrowth was such as to affect the entire synovial membrane and to fill the joint space with new inflammatory tissue. Multiplication of the synovial lining cells, which in their condition of activity resemble fibroblasts was shown in sections by the heaping up of these cells as a loosely arranged covering to the synovial villi several layers in depth. These cells which possess a long axis, generally lay in a roughly radial arrangement around the villus, their free ends often buried in an eosinophilic mass of fibrinous material. Lymphocytes and plasma cells frequently lay between the proliferated synovial cells. They were also scattered throughout the deeper tissues and formed small focal collections sometimes arranged perivascularly. Polymorphs were rarely seen, eosinophils were not infrequent. Vascularity of the synovial tissues was a marked feature even in the earliest case. Villi which were clearly of new formation possessed abundant thin walled blood vessels. The stroma of the villi consisted of new young connective tissue which sometimes showed oedematous myxoid or fibrinoid change. The appearance of the synovial membrane may accurately be likened to that of granulation tissue.

Later changes seen in arthritis of long duration were growth of vascular pannus, destruction of articular cartilage, fibrosis of the deeper synovial tissues and increased lymphoid cell infiltration. Fibrosis and lymphoid cell infiltration of the subarticular epiphyseal bone spaces were also sometimes observed. Pannus, i.e. vascular granulation tissue covering the articular cartilage, was derived from marginal synovial tissue and was often incorporated with proliferated perichondrium. Deformity of the bony epiphyses resulting from collapse or interference with growth was seen in the later stages of the disease. Enlargement of the bones forming the joint was generally due to this process rather than to osteophytic outgrowths and none of the joints resembled those in human osteoarthritis. The infiltration of the synovial tissues with lymphocytes and plasma cells increased with the duration of the arthritis and in our most chronic case of 9 months' duration it had reached enormous proportions, many of the synovial villi containing large lymph follicles which, however, did not possess germinal centres. Joint effusion was present in some cases, absent in others. It was turbid but never frankly purulent. In two animals tenosynovitis was also present in tendon sheaths which communicated with affected joints. Bacteriologically pig 12 stands out as an exception for it died of pneumococcal septicemia and there were foci of pneumococcal

infection in the lungs and subcutaneous tissues. In minor respects the arthritis in this animal differed somewhat from that in the others; there was ulceration and undermining of articular cartilage in the elbow joint, with a large effusion containing considerable numbers of polymorphs as well as small fibrin clots. The histology of the synovial membrane, however, did not resemble that of suppurative arthritis. The inflammatory changes were similar to those found in other animals and cultures from the joint were sterile. It is probable that the pneumococcal septicæmia was a terminal event and ætiologically unrelated to the arthritis.

In many animals there was cultural and serological evidence of infection by *E. rhusiopathiæ*. This organism was recovered from two animals, in pig A from the pus of the empyema and mediastinitis, in pig B from a joint.

Description of micro-organisms from pigs A and B

Strain A. From empyema pus of pig A. Small Gram-positive bacillus. Scanty growth of minute dewdrop colonies on agar. More prolific growth on fresh and heated blood agar, but colonies still very small (about 1.5 mm.). Slight but definite peroxide formation at 72 hours. No catalase production. Moderately good growth on Loeffler's medium. Acid but no gas in glucose and lactose. In broth it produced a fine turbidity with a little granular deposit at the bottom. In gelatin, growth took place along the needle track, with some branching later; no liquefaction. A dose of 0.1 c.c. of an 18-hour culture injected intraperitoneally into mice was lethal within 5 days; the same dose administered intramuscularly killed pigeons in 8-10 days. The organism was agglutinated by swine erysipelas antiserum at dilutions up to 1:3200.

Strain B. Isolated from left radio-carpal joint of pig B. Cultural characteristics were identical with those of strain A, except that acid but no gas was produced in glucose, dulcitol and sucrose. Swine erysipelas antiserum agglutinated the organism at 1:6400.

Indirect evidence of swine erysipelas infection in some animals was furnished by agglutination tests with three strains of *E. rhusiopathiæ*.

Schoening, Creech and Grey (1932), Schoening and Creech (1933, 1935, 1936) and Schoening, Gochenour and Grey (1938) have studied the value of the agglutination test in the diagnosis of swine erysipelas. They, together with Deem (1936), have emphasised the necessity of using a smooth culture in the preparation of the antigen. They considered agglutination at 1:100 or more as evidence of infection. In view of our own results, it is interesting that titres greater than 1:500 were very rarely encountered by these workers, even in pigs repeatedly inoculated with cultures. Schoening and his co-workers pointed out that the agglutination test is not of absolute value in diagnosis in individual animals but that it is of great use in tracing evidence of the infection in herds.

PLATE XXXVII

- FIG. 4.—*Pig A.* Articular cartilage of astragalus with adherent pannus formed from synovial membrane and proliferated perichondrium. H. and E. $\times 15$.
- FIG. 5.—*Pig A.* Gram-positive bacilli resembling *E. rhusiopathia* near surface of synovial tissue of left wrist. No cultures made. Gram. $\times 1300$.
- FIG. 6.—*Pig A.* *E. rhusiopathia* and *Str. haemolyticus* in pleura. Empyema was present. Gram. $\times 1300$.
- FIG. 7.—*Pig A.* Parenchymatous necrosis of heart muscle with little cellular reaction. H. and E. $\times 90$.
- FIG. 8.—*Pig B.* Right elbow joint (on left) arthritic; sigmoid notch of ulna shows proliferation of synovial tissue, erosion of cartilage and widening of articular surfaces due to general hyperplasia of epiphyseal bone. Left elbow (on right) healthy.

POLYARTHRITIS OF SWINE



FIG 4



FIG 5



FIG 6



FIG 7



FIG 8

Marsh (1933) also considered a titre of 1 : 100 as significant. He produced arthritis in lambs by intravenous injection of or exposure of open wounds to cultures of *E. rhusiopathiae* and animals which developed arthritis gave positive agglutination tests. Harrington (1933), working with the rapid slide agglutination test with whole blood described by Schoening and Creech, recorded positive results in a number of pigs with enlarged joints. Pullar (1938) found agglutination titres of 1 : 320 and 1 : 160 in two arthritic pigs. At the time of death of these animals Pullar reported titres of 1 : 7680 and 1 : 15,360, but we have not heard elsewhere of such high titres in this disease.

A single estimation in pig A gave agglutination at 1 : 320 and infection with *E. rhusiopathiae* was proved *post mortem*. In pig B titres ranged from 1 : 25 to 1 : 320 and in pig C from 1 : 20 to 1 : 160. Low titres were found at some periods when these animals were suffering from active arthritis and it may be argued that had the arthritis been due to swine erysipelas infection the titres would have remained significantly high (*i.e.* above 1 : 100). This argument is untenable in view of the findings in pig B. We must assume, therefore, that the agglutination titre fluctuates during the course of chronic swine erysipelas. The experiments to be described later prove this assumption to be correct.

The findings in pigs D 2 and D 3 are interesting in view of the possibility that these animals may have acquired swine erysipelas infection by contact with the arthritic pigs B and C. Following the onset of arthritis, the titre of D 2 increased from 1 : 40 to 1 : 160 but no significant increase took place in D 3.

If we accept a titre of 1 : 100 as significant we have evidence of swine erysipelas infection in C and D 2 as well as in A and B, in which there was direct bacteriological proof of infection. We do not feel justified in excluding swine erysipelas from the other animals on the ground of their low agglutination titres. It is unfortunate that we were unable to make the test at the time when the first three pigs (M, Ma 1 and Ma 2) were examined. In these we have no clue to the aetiology of the arthritis. Cultures of blood, spleen and joints were sterile.

Infection by *Br. abortus*, *melitensis* and *swis* was excluded in certain animals by the appropriate agglutination tests. A high streptococcal antihæmolyisin content was observed in the blood of some animals from time to time. The role of streptococcal infection in swine arthritis will be discussed later but we may state here that we do not consider it to have been important in the cases with which we have dealt.

EXPERIMENTAL OBSERVATIONS

Our observations thus far had confirmed the view held by previous authors that the naturally occurring disease was frequently associated with swine erysipelas. It was known that arthritis

could be induced in swine (Ward, 1922 ; Creech, 1933) and in lambs (Marsh, 1933) by inoculation with *E. rhusiopathiae*, but the pathology of the resulting lesions had not been described. In order to determine the pathological nature of experimental arthritis and to observe the course of development of the lesions cultures of *Erysipelothrix* were injected subcutaneously or intravenously into 12 healthy swine.

Experimental inoculation of swine with E. rhusiopathiae

(a) *By subcutaneous injection.* Two animals (R 1 and L 1) were injected subcutaneously with 5 c.c. of a 24-hour broth culture of *E. rhusiopathiae* and two others (R 2 and L 2) with two similar doses at a fortnight's interval. No evidence of arthritis was subsequently found at autopsy. It must be noted, however, that the interval between the last injection and death was only 1 week. Later experience showed that an incubation period of about 7 weeks generally preceded the appearance of arthritis. Small abscesses were consistently found at the site of injection and it is of interest that, whereas polymorphs predominated in the pus, the leucocytes in the walls of the abscess were almost exclusively monocytes and lymphocytes. Foci of necrosis were found in the liver in two animals and a focus of myocardial necrosis in a third.

(b) *By intravenous injection.* Each of 8 animals was given two or three intravenous injections of *E. rhusiopathiae* broth cultures, preceded in four cases by subcutaneous injections. All subsequently developed polyarthritis as well as lesions of internal organs. Cultures were taken from the blood and joints from time to time during life and *post mortem* and the serum agglutination titre against *E. rhusiopathiae* was frequently estimated. Streptococcal antihæmolyisin titrations were also made repeatedly. These showed no variation from the normal in the pigs of litter G and have therefore been omitted from the protocols of this group.

Fig D 4. Hog

Age (weeks)	Clinical arthritis	Aggl. of <i>E. rhus.</i>	Antihæmolyisin M.H.D.	Remarks.
8	0	...	480	...
9	5 c.c. <i>E. rhus.</i> (A) culture subcutaneously
13	0	1:20	480	...
16	0	...	200	...
22	1 c.c. <i>E. rhus.</i> (A) culture intravenously	No signs of arthritis
24	5 c.c. " " "	" " "	" "	...
25	++	" " "	" " "	Swollen joints and lameness

Post-mortem examination. Moderately severe arthritis of right knee, right wrist, right and left shoulders and right and left elbows. Gram-positive organisms seen in sections of sub-synovial tissue of right shoulder (fig. 14). Enlargement of mesenteric glands. Inflammatory arteritis of vessels of

PLATE XXXVIII

- FIG. 9.—*Pig B.* Hypertrophied synovial villus from left shoulder diffusely infiltrated by lymphocytes. H. and E. $\times 110$.
- FIG. 10.—*Pig B.* Head of femur showing (from without inwards) perichondrium, cellular cartilage, new bone, fibrous tissue, original articular cartilage and original bone. This formation may have resulted from chondrification and ossification of adherent pannus. Hematoxylin and van Gieson. $\times 30$.
- FIG. 11.—*Pig C.* Synovial membrane from right ankle, showing increased fibrosis, slight proliferation of lining cells and lymphocytic infiltration around deeper blood vessels. H. and E. $\times 110$.
- FIG. 12.—*Pig E.* Focus of fibrinoid necrosis from right elbow surrounded by radially arranged fibroblasts in the deeper synovial tissues. Lymphocytic infiltration. H. and E. $\times 60$.
- FIG. 13.—*Pig E.* Smaller irregular focus of fibrinoid necrosis with aggregation of histiocytes in deep connective tissue of capsule of right elbow. H. and E. $\times 140$.
- FIG. 14.—*Pig D 4.* *Erysipelothrix* in subsynovial muscle of right shoulder. Arthritis from repeated injections of *E. rhusiopathiae*. Gram. $\times 1300$.

POLYARTHRITIS OF SWINE

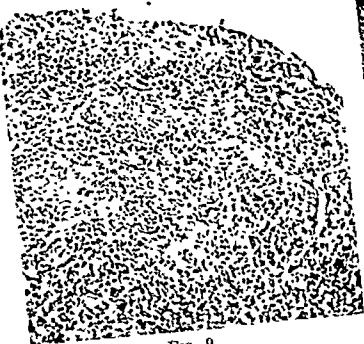


FIG. 9.



FIG. 10.



FIG. 11.

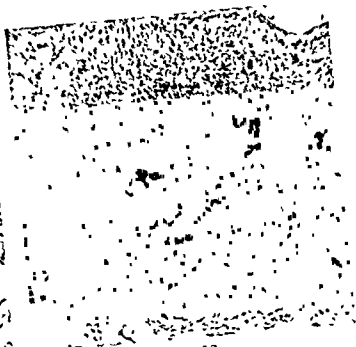


FIG. 12.

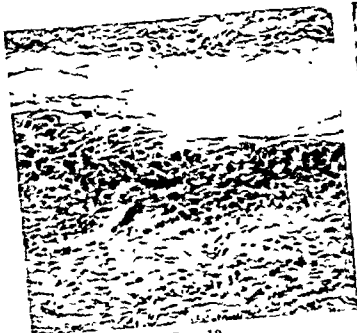


FIG. 13.



FIG. 14.

heart and kidneys (figs 15, 16, 17 and 18) *Erysipelothrix* recovered from spleen and right shoulder. Cultures from heart blood, right knee and right elbow sterile. Joint fluid from left elbow injected into pigeon without effect

Fig F2 Gilt

Age (weeks)	Clinical arthritis	Aggl of <i>E. rhus</i>	Antihemolysin M H D	Remarks
10	Vaccinated with swine erysipelas vaccine + antiserum			Cohabited with arthritic pig B from 10th to 19th week but did not develop arthritis
11	0		180	
"	0.5 cc live swine erysipelas vaccine subcutaneously			
13	0	1 200	200	
19	5 cc <i>E. rhus</i> (A) culture		intravenously	Appears somewhat ill
21	5 cc "	" "	" "	
25	0			
"	7 cc <i>E. rhus</i> (A) culture		intravenously	
31	0	1 100	1280	Local abscess only Slight limping Arthritis more severe Some recovery
"	5 cc <i>Str. ham</i> (pig) culture		subcutaneously	
34	+			
47	++			
50	±			Has suffered a definite relapse
52	10 cc <i>E. rhus</i> (B) culture		intravenously	
	+			
55	Killed			

Post mortem examination Moderately severe arthritis of all main limb joints except left shoulder. The microscopic changes in the joint tissues include widespread fibrinoid degeneration of stroma of synovial villi (fig 20). Enlargement of mesenteric glands. *Erysipelothrix* recovered from six joints. Sterile cultures from heart blood and two joints.

Fig F3 Gilt

Age (weeks)	Clinical arthritis	Aggl of <i>E. rhus</i>	Antihemolysin M H D	Remarks
11	0		180	Cohabited with arthritic pig B from 10th to 19th week but did not develop arthritis
13	0	1 25	50	
19	5 cc <i>E. rhus</i> (A) culture		intravenously	
21	5 cc "	" "	" "	
25	0	1 800	" "	Local abscess only Limping A relatively mild arthritis
"	7 cc <i>E. rhus</i> (A) culture		intravenously	
31	0	1 800	640	
"	5 cc <i>Str. ham</i> (pig) culture		subcutaneously	
34	+			Killed
39	+		960	
40				

Post mortem examination Moderate arthritis of many joints. Focal collections of lymphocytes prominent microscopically in joint tissues (fig 21).

Erysipelothrix recovered from right wrist and right elbow. Sterile cultures from heart blood, spleen, left wrist and left and right ankles.

Fig F4. Hog

Age (weeks)	Clinical arthritis	Aggl. of <i>E. rhus.</i>	Antihæmolytic M.H.D.	Remarks
11	0	...	90	Cohabited with arthritic pig B from 10th to 19th week but did not develop arthritis
13	0	0	1600	...
19	5 c.c. <i>E. rhus.</i> (A) culture	intravenously		...
21	5 c.c. " " "	"	"	...
25	7 c.c. " " "	"	"	...
31	0	1 : 800	1280	...
"	5 c.c. <i>Str. hæm.</i> (pig) culture	subcutaneously		Local abscess
34	+	1 : 800
35		Died		...

Post-mortem examination. Moderate arthritis of all main limb joints except right shoulder and left hip. Focal collections of lymphocytes in synovial tissues a marked feature (figs. 22 and 23). Infective endocarditis (fig. 24). Foci of myocardial necrosis. White infarct of kidney. Bilateral pleural effusion and recent pleurisy. *Erysipelothrix* recovered from blood, endocardial vegetations, spleen, pleural effusion, left wrist, left elbow and left knee. Cultures from right ankle and liver sterile.

Fig G1. Gilt

Age (weeks)	Clinical arthritis	<i>E. rhus.</i> in joint culture	Aggl. of <i>E. rhus.</i>	Remarks
9	0	...	1 : 25	...
"	5 c.c. <i>E. rhus.</i> (B) culture	subcutaneously		...
11	0	...	1 : 50	...
"	2 c.c. <i>E. rhus.</i> (B) culture	intravenously		...
13	0	...	1 : 400	Coughing
"	2.5 c.c. <i>E. rhus.</i> (B) culture	intravenously		...
15	0	...	1 : 400	...
19	+	Early arthritis
23	+	...	1 : 200	Stiff legs; arched back
28	+	L. knee + R. ankle -
31	+	L. knee - L. wrist -	...	Blood culture sterile; arthritis increasing
37	++	Now much crippled; blood culture sterile
39	+	L. knee - L. wrist -	1 : 100	More active; joints re-covering
42	±	Killed

Post-mortem examination. Mild arthritis of several joints. Old vascular changes in vessels of heart (fig. 19). Cultures from heart blood, spleen and six joints sterile.

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PLATE XXXIX

- FIG. 15.—*Pig D 4*. Arteritis of a main coronary artery branch. Intimal hyperplasia, medial degeneration and adventitial fibrosis, with lymphocytic infiltration of adventitia and part of media. H. and E. $\times 110$.
- FIG. 16.—*Pig D 4*. Small branch of a coronary artery showing periarterial collection of lymphocytes. H. and E. $\times 140$.
- FIG. 17.—*Pig D 4*. Arteritis of vessel in kidney substance. Replacement of media by hyaline. Concentric arrangement of fibroblasts causing great increase in thickness of adventitia, which is heavily infiltrated by lymphocytes and plasma cells, together with a few eosinophils and polymorphs. H. and E. $\times 140$.
- FIG. 18.—*Pig D 4*. Arteritis of vessel in kidney substance. The oblique section clearly shows focal nature of lesion and gradual replacement of muscular wall by hyaline. H. and E. $\times 110$.
- FIG. 19.—*Pig G 1*. Heart. Coronary artery branch showing intimal hyperplasia and increase of adventitial fibrosis in animal partially recovered from effects of *Erysipelothrix* infection $5\frac{1}{2}$ months previously. Elastic tissue stain. $\times 60$.

POLYARTHRITIS OF SWINE

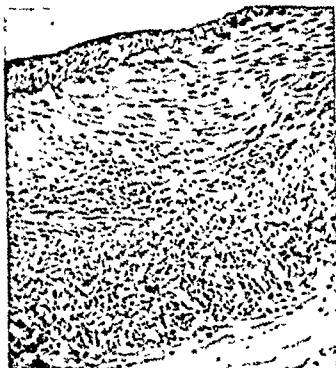


FIG. 15.



FIG. 16.

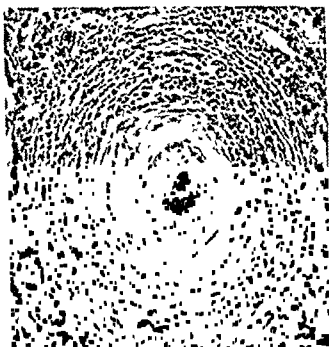


FIG. 17.



FIG. 18.



FIG. 19.

Fig G 4. Gilt

Age (weeks)	Clinical arthritis	<i>E. rhus.</i> in joint culture	Aggl. of <i>E. rhus.</i>	Remarks
9	0	...	0	...
"	2 c.c. <i>E. rhus.</i> (B) culture	intravenously
11	0	...	1 : 50	...
"	2 c.c. <i>E. rhus.</i> (B) culture	intravenously
13	0	...	1 : 400	...
"	2.5 c.c. <i>E. rhus.</i> (B) culture	intravenously
15	0	...	1 : 50	...
22	+	...	1 : 200	Arthritis apparent
27	++	L. elbow + L. wrist +	...	Severe arthritis
32	+++	L. knee +	...	Emaciated and frail
33		Died		...

Post-mortem examination. Moderately severe arthritis of knees, ankles, elbows and wrists. Enlargement of inguinal and mesenteric lymph nodes. Focal necrosis of mid-zone of many liver lobules (fig. 29). Suppurative cholangitis. *Erysipelothrix* recovered from left knee, left elbow and right ankle. Sterile cultures from heart blood, both wrists, right elbow, left ankle and right knee.

Discussion of experimental work with *E. rhusiopathiae*

Arthritis

Polyarthritis developed in all the 8 animals which had received intravenous injections of *E. rhusiopathiae*. In 4 animals intravenous injections only were given and in the other 4 these were preceded by a subcutaneous inoculation. Both strains used proved equally successful in inducing arthritis. Subcutaneous inoculations alone failed to produce arthritis but the animals concerned may not have been kept sufficiently long under observation.

The 8 animals developing arthritis died or were killed at intervals ranging from 1 to 27 weeks after the onset of arthritis.

Clinical course. The onset of arthritis was always gradual and in all except one animal (D 4) there was a delay of 6-9 weeks between the last injection and the clinical appearance of arthritis (table II). Accurate estimation of this incubation period was difficult, as the earliest clinical signs were not easily noticed. We do not yet know whether this period was the time required for the organisms to localise in the joints or for the pathological changes to reach sufficient severity to cause symptoms. The total dose of bacteria injected and the number of inoculations varied (table II). The largest single injection given before the onset of arthritis was 7 c.c. of an 18-hour broth culture. One animal (F 2) was given an injection of living organisms when its arthritis seemed to be remitting and this undoubtedly caused a relapse. Another (G 3) received an intravenous injection of killed organisms after arthritis had been manifest for 24 weeks but this did not appear to influence the course of the disease.

The disease attained its maximum severity 6-8 weeks after it had first become manifest. At this stage the animals were severely crippled, stunted and emaciated. They preferred to remain lying down. When they were made to stand, deformities such as arched back, scissors gait or flexed wrists

12

13

PLATE XL

- FIG. 20.—*Pig F 2*. Experimental *Erysipelothrix* arthritis. Fibrinoid degeneration in hypertrophied synovial villus. Phosphotungstic acid hæmatoxylin. $\times 10$.
- FIG. 21.—*Pig F 3*. Synovial membrane of right knee in experimental arthritis. Enlarged and highly cellular synovial villi with focal collections of lymphocytes. Collection on right is centred around a blood vessel. H. and E. $\times 60$.
- FIG. 22.—*Pig F 4*. Synovial membrane of left knee in induced arthritis. Large focal collection of lymphocytes in synovial villus. H. and E. $\times 60$.
- FIG. 23.—*Pig F 4*. Detail of lymphocytic collection in synovial membrane shown in preceding figure. H. and E. $\times 160$.
- FIG. 24.—*Pig F 4*. Endocarditis of mitral valve due to *Erysipelothrix*.

POLYARTHRITIS OF SWINE



FIG 20



FIG 21



FIG 22

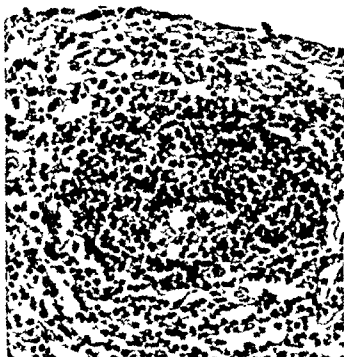


FIG 23



FIG 24.

could be seen, as well as swollen joints (fig 1 d), and movements were painful and accompanied by much squealing. Swelling of the joints at this time was due to intra articular effusion and inflammatory œdema of the soft tissues. This phase of the disease lasted for many weeks. Two animals

TABLE II
Number of injections and total doses of *E. rhusiopathiæ* and incubation periods before appearance of arthritis

Animal	Number of injections		Aggregate dose of live cultures	Time of onset of arthritis after	
	Subcutaneous	Intravenous		first injection	last injection
D 4	1	2	11 0 c c	16 weeks	1 week
F 2	1	1	17 5 "	23 "	9 "
F 3	0	3	17 0 "	15 "	9 "
F 4	0	3	17 0 "	15 "	9 "
G 1	1	2	9 5 "	10 "	6 "
G 2	1	2	9 5 "	13 "	9 "
G 3	0	3	6 5 "	10 "	6 "
G 4	0	3	6 5 "	13 "	9 "
Average				14 4 weeks	7 3 weeks

(G 1 and G 2) began to recover after having had severe arthritis for 20 and 17 weeks respectively. Two others were ill for 22 and 27 weeks and were then killed and at autopsy still showed active lesions in the joints. Two pigs died but lesions in organs other than the joints were held to be responsible for their deaths, in one (F 4) infective endocarditis and in the other (G 4) a debilitated condition and early suppurative cholangitis.

Pathology The earliest lesions, as seen in pig D 4 a week after the clinical appearance of arthritis, consisted of inflammatory œdema and vascular engorgement of the synovial tissues and joint capsule, with slightly turbid, mucinous effusion into the joint. The synovial villi showed only slight cellular proliferation and sparse lymphocytic infiltration. *E. rhusiopathiæ* was isolated from one joint showing these changes and the organisms were seen microscopically lying in groups in the deeper tissues at the edge of the capsular muscle (fig 14). Cultures from two similarly affected joints proved sterile and no organisms were seen in sections.

In arthritis of longer duration there was less œdema and greater cellular proliferation. The synovial lining cells had multiplied and formed a loosely arranged covering to the overgrown synovial villi several cell layers in depth (fig 21). The villi were increased both in size and in complexity and were formed of young, highly vascular connective tissue infiltrated with lymphocytes and plasma cells. These infiltrating cells were usually most numerous immediately beneath the synovial lining, where they often formed focal collections. Dense foci of lymphocytes were a striking feature of many of the joints (figs 21 and 22). Their appearance differed

from that of the follicles of lymphatic glands in that they did not possess germinal centres (fig. 23). Though sometimes perivascular in arrangement in the deeper tissues, this was not the rule within the synovial villi. Many villi, particularly in the more chronically affected joints, resembled granulomatous polyps in structure (figs. 26 and 28), but polymorphs were seldom found in them and the majority of leucocytes were lymphoid or plasma cells, with a scattering of eosinophils. Some sections revealed minute areas of suppuration (fig. 27). These were small ulcers of the synovial membrane filled by typical granulation tissue and fibrinous exudate containing many polymorphs and erythrocytes. These appearances were seen only in joints from which organisms were recovered in culture. Suppuration was never of more than microscopic extent and most of the tissue in such sections always showed proliferative and chronic inflammatory changes. It would seem that the destructive and suppurative type of reaction is alternative to the proliferative and lymphocytic and that the one merges into the other. Which type of change predominates may depend upon such factors as the intensity of the local infection or the time of survival of the bacteria *in situ*. We never observed the extensive destruction of cartilage and bone which characterises suppurative arthritis in other species. Synovial effusions were non-purulent. They contained polymorphs, lymphocytes, monocytes, macrophages and synovial lining cells. Polymorphs rarely exceeded 50 per cent. of the cells and the total nucleated cell count was never very high. In general, the effusions cytologically resembled those occurring in human rheumatoid arthritis (Collins, 1936). These pathological features together with the clinical course of the disease served to distinguish it from frank pyæmic arthritis.

Inflammatory reaction in the sub-chondral bone consisting of fibrosis of the marrow, increased vascularity and the presence of small collections of lymphocytes was observed in some joints. Pannus formation and superficial erosion of cartilage at the sites of pannus attachment were also not infrequently seen, and in the later stages of the disease there were sometimes intra-articular fibrous adhesions and fibrosis of the joint capsule.

Permanent enlargement of the joints in the late stages of the disease was due to increase in size of the bone ends or, in the tarsus and carpus, to overgrowth of the small bones as a whole and to the laying down of new periosteal bone. Irregular periosteal ossification was clearly shown in X-ray photographs of some limbs. In other joints collapse and splaying of the articular epiphyses had taken place. Marginal osteophytes similar to those in human osteoarthritis were not observed. The unusual structural alterations of bone in these animals may have been influenced by the fact that they were young pigs whose epiphyses were not yet united.

12

2

3

4

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6

7

8

9

10 11 12

13

14

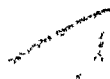


PLATE XLI

- FIG. 25.—*Pig G 2*. Synovial villi of left shoulder showing a succession of obliterated blood vessels in animal partially recovered from arthritis induced by infection with *Erysipelothrix* 5 months previously. Hæmatoxylin and van Gieson. $\times 60$.
- FIG. 26.—*Pig G 3*. Induced *Erysipelothrix* arthritis in left knee of 6 months' duration. Patella reflected upwards. Condyles of femur towards bottom of picture. Joint filled with enlarged synovial villi. Above patella is an area of roughened ulcerated synovial membrane.
- FIG. 27.—*Pig G 3*. An area of the synovial surface illustrated in figs. 26 and 28. Ulceration of surface with deposit of fibrin containing numerous polymorphs. Organisation is commencing from beneath. H. and E. $\times 60$.
- FIG. 28.—*Pig G 3*. Enlarged vascular synovial villus from left knee joint resembling a granulomatous polyp. Infiltrating cells almost entirely lymphocytes. H. and E. $\times 60$.
- FIG. 29.—*Pig G 4*. Focal necrosis of liver parenchyma and cellular reaction in the mid-zone of a lobule. Experimental *Erysipelothrix* infection. H. and E. $\times 60$.

POLYARTHRITIS OF SWINE

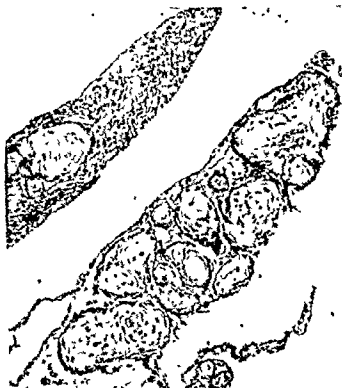


FIG. 25



FIG. 26



FIG. 27.

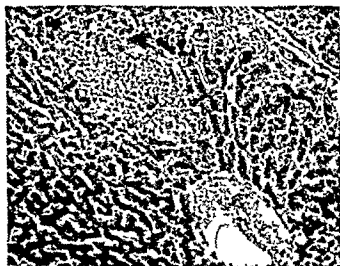


FIG. 29

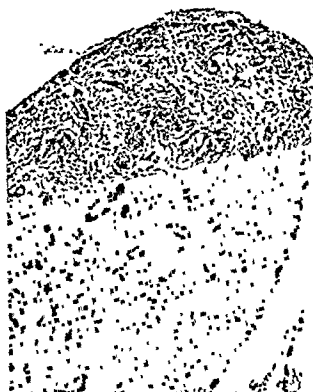


FIG. 28.

Spontaneous recovery or remission of the arthritis commenced in two animals before they were killed and the joints examined. Remarkably little change was found in certain of the joints which were known to have been grossly affected during the active phase of the disease. Signs of inflammation had generally disappeared and there was often little to be seen either with the naked eye or microscopically, but other joints which must have undergone greater intra articular damage were found to contain fibrous adhesions and incompletely healed erosions of cartilage. Microscopical evidence of resolution consisted of fibrosis of the synovial tissues and obliterative endarteritis in previously richly vascular villi (fig 25). Cultures from these recovered joints were uniformly sterile.

Bacteriology In all, 73 cultures were taken from the joints of arthritic animals during life or at autopsy. Of these 25 (34 per cent) yielded *E. rhusiopathiae*. Their relation to the injections and to the results of blood and splenic cultures is shown in table III.

TABLE III

Summary of bacteriological cultures during life and at autopsy in animals developing experimental *Erysipelothrix* arthritis

Fig	Total number of injections of <i>E. rhusiopathiae</i>	Interval in weeks since		<i>E. rhusiopathiae</i> in cultures from			Remarks
		first in injection	last in injection	blood	spleen	joints	
D 4	3	16	1	—	+	+ 1 — 3	Post mortem
I 2	5	44	5	+		6 2	Post mortem
F 3	3	21	15	—	—	2 3	Post mortem
F 4	3	16	10	+	+	3 1	Post mortem Endocarditis present
G 1	3	10	15			1 1	
		22	18	—		0 2	
		28	24	—			
		30	26			0 2	Arthritis recovering
		33	29	—	—	0 6	Post mortem
G 2	3	17	13			0 2	
		22	18	—		0 1	
		28	24	—			
		30	26			0 2	Arthritis recovering
		34	30	—	—	0 9	Post mortem
G 3	3	18	14			1 1	
		23	19			2 1	
		28	24	—			
		30	26			0 2	
		37	33	—	+	3 5	Post mortem
G 4	3	18	14			2 0	
		23	19			1 0	
		24	20	—		3 5	Post mortem

The summary given in table IV shows how the proportion of positive joint cultures diminished with the passage of time after the injections, the difference becoming significant after the 20th week. Two animals had positive blood cultures and cultures from their joints were more frequently positive (75 per cent.) than those from animals with sterile blood (26 per cent.).

TABLE IV

Effect of time since last injection on result of cultures from joints

Time after last injection	Number of cultures	<i>E. rhusiopathiae</i> present	
		Number	Per cent.
1-10 weeks	16	10	62.5
11-20 "	28	12	42.9
Over 20 "	29	3	10.3

We know that *E. rhusiopathiae* can survive in the tissues for a very long time. The organisms were grown from 3 joints and from the spleen of pig G 3 thirty-three weeks after the last injection and from one joint of pig B thirty-six weeks after the onset of arthritis. Organisms were seen in sections in only one early case of arthritis (D 4) and never in smears of joint fluid stained by Gram's method. Gross suppuration never occurred in the joints. It is probable, therefore, that the joints were not heavily infected. Occasionally anomalous results were obtained from successive cultures of the same joint. Four cultures made at intervals from the left knee of pig G 3 yielded alternate positive and negative results. We feel bound, therefore, to regard positive cultures as more significant than negative. There may well be an element of chance attached to the presence or absence of viable organisms in the synovial fluid at a given time.

An attempt has been made to correlate the presence or absence of bacteria with the extent of the pathological changes in the joints. Joints showing slight changes have been consistently sterile, while the incidence of positive and negative cultures in those showing moderate or severe changes was approximately equal (table V). It would seem therefore that a proliferative arthritis may become sterile without any apparent regression of the pathological changes. This view is supported by our observation in field cases of the disease, where *E. rhusiopathiae* was recovered from only one out of 16 joints, most of which showed gross arthritic changes.

Microscopically, chronic inflammation with lymphocytic reaction was seen in both sterile and infected joints, but the small areas of

phlegmonous ulceration to which reference has been made were found only in joints giving positive cultures.

TABLE V

Relation of cultural results to degree of arthritis in joints examined at autopsy

Degree of arthritis	Number of joints examined	Cultures	
		Positive	Negative
Slight . . .	12	0	12
Moderate .	25	10	15
Severe . . .	15	8	7

E. rhusiopathiae grew in 2 out of 8 post-mortem blood cultures. The persistent bacteriæmia in F 4 can be attributed to the presence of the organisms in the endocardial vegetations. The other positive blood culture was obtained from F 2 which, 5 weeks before killing, had received a large intravenous dose of living organisms and had suffered a relapse of its arthritis.

Three out of 6 splenic cultures were positive 1, 10 and 33 weeks after the last injection. These findings show that the spleen, like the joints, can harbour viable *Erysipelothrix* for long periods. No notable pathological changes, either macro- or microscopic, were seen in these spleens.

Sequence of events in induced arthritis. From our experience we may deduce the following course of events. Bacteria introduced into the blood stream reach the joints and settle in the deeper strata of the synovial membrane, setting up an inflammatory reaction. This takes the form of interstitial cedema, vascular congestion and intra-articular effusion. The organisms disseminate through the synovial tissues, and some pass out into the joint fluid. Proliferative changes occur in synovial lining, villi and connective tissues, and at the same time lymphocytes and plasma cells arrive in large numbers. Polymorphs also invade the tissues, but in smaller numbers, and the majority pass into the joint effusion. Eosinophils are found in the tissues. Suppuration only occurs focally, possibly in areas where bacteria are most numerous. The synovial membrane becomes converted into a sort of granulation tissue and individual villi resemble granulomatous polyps but the synovial lining is rarely lost. New synovial lining cells are probably being constantly developed from underlying fibroblasts. Fibrinoid necrosis of connective tissue may occur (fig. 20). Extension of the synovial fringes over the articular surfaces combined with proliferation of perichondrium forms pannus and the articular cartilage may be

eroded superficially at the points of pannus attachment and deeply by inflammatory reaction in the subchondral bone marrow. About this time the bacteria probably disappear from the joints unless reinforcements arrive by the blood stream from distant reservoirs. When the joints become sterile one of three things may happen, (a) the lesions may resolve and the joints recover, (b) healing of the damaged tissues may take place by fibrosis and this may include the formation of intra-articular adhesions, or (c) the synovial granulation tissue may assume certain properties of a benign neoplasm and persist and extend in the absence of the exciting micro-organisms, thus maintaining the arthritis in a state of clinical activity.

The third possibility is not a new pathological conception, for some such explanation has frequently been mooted in regard to nasal polyps and keloid scars. We cannot claim to have proved this to occur in joints, but our experiments encourage us to entertain the possibility. Such a turn of events would explain the bacteriological findings summarised in table V. This matter has so important a bearing on chronic proliferative arthritis in man that we are continuing to investigate it.

Lesions in organs other than joints

Heart. Typical infective endocarditis with large friable vegetations on the mitral valve occurred in one animal (F 4, fig. 24). The vegetations contained numerous *E. rhusiopathiae*. Endocarditis is thought to be a commoner chronic form of swine erysipelas than arthritis (Hutyra, Marek and Manninger, 1938). It is not infrequently found in carcasses at abattoirs. In an affected heart sent to us from an abattoir we found appearances identical with those seen in the experimental animal. Focal parenchymatous degeneration of the myocardium with cellular reaction and early replacement fibrosis was found in two animals (F 4 and L 1) experimentally infected, and three times in field cases of arthritis (fig. 7). Pericardial effusion did not occur in the experimental infections though it was common in field cases.

Arteries. A peculiar inflammatory arteritis was observed in some experimental animals (figs. 15-19). As far as we are aware this has not been described before. The arteritis in its acuter stages bears some resemblance both to polyarteritis nodosa and to rheumatic arteritis of man. In the acute phase there are swelling of the intimal endothelium, hyaline degeneration of the media and perivascular collections of lymphocytes and fibroblasts. These appearances were seen in two animals (D 4 and G 4). Lesions which were interpreted as representing the healing phases of an inflammatory arteritis were seen in three animals (F 2, G 1 and G 2)—intimal fibrosis, replacement of medial muscle fibres by

collagen and great thickening of the adventitia. The intimal changes did not resemble atheroma except that they were patchy in distribution. Neither thrombosis nor aneurysm formation was observed. The lesions were found microscopically in the vessels of heart, kidney, mesentery and synovial membrane. They were not systematically sought for. No such lesions were seen in control animals or in animals infected with streptococci.

Liver Foci of parenchymatous necrosis accompanied by cellular reaction were seen in the mid-zone of the liver lobules in four experimentally infected animals (R 2, L 1, F 3, and G 4) (fig. 29). Gram-stained sections did not reveal any organisms. Barber (1939) remarks on the occurrence of focal necrosis of the liver in rabbits and mice injected with *Erysipelothrix*. Both in *Erysipelothrix* and *Listerella* infections the liver necrosis seems to be a sequel of bacteraemia. An inflammatory cell reaction in the neighbourhood of the portal tracts was found in two of our pigs. In F 2 this consisted mainly of eosinophil granulocytes but in G 4 the lesion was clearly a suppurative cholangitis, although liver abscesses were too small to be visible to the naked eye.

Lymph glands The regional lymph nodes were sometimes enlarged in animals with arthritis of the hind limbs. In four animals (F 2, F 3, G 2 and G 4) there was marked enlargement of the mesenteric glands and this was a feature also of many of our field cases. Microscopically the enlargement was due to hyperplasia of all the glandular elements and there was invariably marked infiltration with eosinophil leucocytes. Organisms were not seen nor were they isolated from the field cases in which cultures were made. The condition was not observed in non-arthritic animals, but it was not possible to exclude local—particularly parasitic—disease of the intestines.

Blood Barber (1939) found that *Erysipelothrix* like *Listerella* infection gives rise to a circulating monocytosis in rabbits, and Egehoj (1937) showed that swine erysipelas is accompanied by a mononuclear leucocytosis with a neutrophil leucopenia. He also described the appearance in the blood of atypical large mononuclear lymphoid cells. We have made total or differential leucocyte counts in a number of animals. Table VI shows the leucocytic picture in two field cases of arthritis, in three pigs 3 days to 7 weeks after a single subcutaneous injection of *E. rhusiopathiae*, and in three pigs suffering from arthritis 6 months after intravenous injections. The average normal blood picture of young and adult pigs (Fraser, 1938) and the results of blood examinations made on healthy bacon pigs at a slaughter house are also given for comparison. The absolute numbers of monocytes are shown in the last column. There was a significant increase in the number of circulating monocytes in both field cases of arthritis and in two of the animals

subcutaneously infected with *E. rhusiopathiae*. There was also a slight increase of these cells in the animals which had developed arthritis following intravenous inoculations of *Erysipelothrix*.

TABLE VI
Total and differential blood leucocyte counts

Animal	Condition of animal	Total W.B.C. per c.mm.	Differential percentages					Absolute no. of monocytes per c.mm.
			P.	E.	B.	L.	M.	
E	Field case of arthritis	19,800	20	7	...	47	26	5150
D 2	" " "	19,800	22	1	1	56	20	3960
L 1	3 days after <i>E. rhus.</i> subcutaneously	48,000	23	1	...	67	9	4320
L 2	" " " "	35,000	19	1	...	76	4	1400
D 1	7 weeks after <i>E. rhus.</i> subcutaneously	19,400	24	...	1	56	19	3690
G 1	6 months after <i>E. rhus.</i> intravenously. Ar- thritis+	14,000	47	4	2	34	13	1820
G 2	" " " "	13,500	33	3	1	49	14	1890
G 3	" " " "	14,000	34	4	1	44	17	2380
I	Healthy bacon pig at slaughter	...	52	1	1	41	5	...
II	" " " "	...	57	39	4	...
III	" " " "	...	28	2	...	65	5	...
Average of 11 pigs*	Healthy: aged 2-7 months	21,000	40	6	...	48	6	1260
Average of 15 pigs*	Healthy: aged 1½-2 years	15,000	53	4	...	39	4	600

* Figures from Fraser (1938).

Skin. Skin manifestations as seen in acute swine erysipelas were never observed either in experimental or in natural arthritis. Our observations bear out the opinion of Craig (1926) and Creech (1937) that the chronic arthritic form is not necessarily preceded by the acute disease. In two animals, however, which we used for the contact experiment (D 2 and D 3) arthritis developed acutely within 4 and 10 days respectively, but here again there were no skin manifestations. The position is not that it is impossible to induce acute swine erysipelas experimentally, for Creech (1933) occasionally succeeded, but that we have in our experiments unknowingly reproduced the conditions which favour the development of the chronic rather than of the acute disease.

Serum agglutinins. The agglutination titre of the pigs' sera against *E. rhusiopathiae* generally rose a few weeks after infection but frequently fell again even after repeated injections of bacteria (see protocols). Low titres were sometimes found in the pre-ence of active arthritis and at times when we knew the animals harboured

living bacteria. Fluctuations in the titre in individual animals were also observed and it would appear that the test is not of diagnostic value except where increased titres are observed. Titres below 1 : 100 as found in two of our field cases do not therefore rule out the possibility of swine erysipelas infection.

COMPARISON OF EXPERIMENTAL WITH NATURALLY OCCURRING ARTHRITIS

Enough has been said to prove that the anatomical changes in experimental arthritis are identical with those encountered in the naturally occurring disease. We are convinced that *E. rhusiopathiae* has been the cause of the spontaneous disease in some of the field cases for reasons already put forward, namely the association of arthritis with proved *Erysipelothrix* infection in two animals, in one of which the organism was grown from a joint, and the high agglutination titre found in two others. One striking difference between the two sets of observations was the frequent isolation of bacteria in joint cultures from experimental animals (25 out of 73 joints positive) and their infrequent isolation in the natural disease (1 out of 16 joints positive). We have already stated our belief that negative joint cultures are not so significant as positive and we have proved that low agglutination titres may occasionally occur in chronic cases of known swine erysipelas infection. It is therefore possible that the arthritis in all the field cases may have been due to *E. rhusiopathiae*; on the other hand it seems probable that other common pathogenic organisms also cause a similar polyarthritis in swine.

The mechanism of the arthritis is an interesting problem. The experimental lesions are certainly bacteraemic. Arthritis in our experimental animals has thus far followed multiple injections of bacteria, and the induction of a condition of hypersensitivity ("hyperergy") seemed a possible factor in the localisation of the organisms in the joints. Creech's (1933) experimental arthritis from single massive injections of *Erysipelothrix* seems to indicate that sensitisation was not essential, but the joints were not pathologically examined and the condition, which was acute, may have been a suppurative pyaemic arthritis. Creech used much larger doses of living cultures and did not produce arthritis so regularly as we did. Neither Creech's nor our own methods resembled anything likely to occur naturally and they do not fully explain the pathogenesis of the natural disease. Marsh (1933) seems to have reproduced the natural conditions more satisfactorily. He exposed new-born lambs and lambs with open docking and castration wounds to bedding heavily infected with cultures of *E. rhusiopathiae*. Arthritis developed in some animals after an interval of 1-11 weeks.

The spontaneous disease in swine frequently starts just after weaning at 8-10 weeks old. If the incubation period up to 9 weeks observed in our experiments also occurs in the natural disease, the onset of arthritis in young swine after weaning may be attributed to infection entering through the umbilical stump or through castration wounds. At the same time the possibility of hypersensitivity and exposure to later infection had not been excluded in Marsh's work, and the rapidity with which arthritic symptoms in our pigs D 2 and D 3 followed contact with arthritic animals suggests such a basis if we assume them to have been already sensitised. It seemed most unlikely that amongst all our animals these were the only two which carried a latent infection at the time of their acquisition by us. The localisation and development of joint lesions might possibly result from the combined effects of tissue sensitivity and repeated temporary bacteraemias.

HYPERSENSITIVITY EXPERIMENT

In order to ascertain what part hypersensitivity plays in swine arthritis, experiments were carried out in six animals (H 1-H 6). This experiment was interrupted by the outbreak of war, but sufficient information was gained to make publication worth while.

A culture of *E. rhusiopathiae* on heated blood agar was suspended in normal saline and subjected to alternate freezing (with liquid CO₂) and thawing until no bacteria could be recognised in a smear. The suspension was centrifuged at 3000 *r.p.m.* for an hour, the clear supernatant fluid pipetted off and 0.5 per cent. phenol added. The non-protein nitrogen of the autolysate was estimated at 35.1 mg. per cent. One-half c.c. of a 1 : 50 dilution of this was injected intradermally into the ear of each pig. As no erythema or thickening had been observed up to 48 hours it was presumed that no sensitivity to *E. rhusiopathiae* existed in these animals. Successive doses of 0.5 and 1.5 c.c. of the 1 : 50 dilution and 0.5 and 1.5 c.c. of the original autolysate were injected at 4-day intervals into the ear veins of three pigs (H 1, 2 and 3). One week after the last injection skin tests were carried out as before on all pigs. Forty-eight hours after this second test there was seen at the site of injection in pigs H 1, 2 and 3, an area of erythema and thickening about 1 cm. across. Hypersensitivity to the autolysate seemed therefore to have been established in these animals. No reaction was observed in the control pigs (H 4, 5 and 6).

All six pigs were then given a single intravenous dose of 2.5 c.c. of a 24-hour broth culture of *E. rhusiopathiae* and observed for 3 months. Although no clinical evidence of arthritis was seen, post-mortem examination revealed the presence of inflammatory joint changes in all six animals.

The gross pathology of the lesions resembled that of the mildest forms of arthritis which we had previously observed. There was increased and frequently slightly turbid effusion into the joints. Inflammatory congestion of the synovial villi was always particularly prominent opposite the inter-joint line and in some of the more severely affected joints enlarged and inflamed synovial villi were present. Destructive cartilaginous changes were absent. Eight joints examined were bacteriologically sterile. The microscopic and macroscopic appearances assured us that the arthritis was similar to that recorded in our previous experiments and of such a type that recovery would have taken place.

COMPARISON OF SWINE ARTHRITIS WITH RHEUMATOID ARTHRITIS IN MAN

The disease which we have observed is a chronic proliferative arthritis bearing a general resemblance to rheumatoid arthritis of man and to certain other specific forms of chronic inflammatory arthritis, *e.g.* gonorrhœal. In so far as the clinical appearances of a disease can be compared in two different species, the course of the swine arthritis in its gradual onset and prolonged activity, followed either by resolution or by permanent interference with function, seems to correspond to the infective or rheumatoid forms of chronic arthritis in man. The anatomical changes in the joints also show clear macroscopic and microscopic similarities. The lesions are mainly non-suppurative and comprise granulomatous proliferation of the synovial membrane, pannus formation and infiltration of the tissues with lymphocytes, which often form subsynovial focal collections. Intra-articular fibrous adhesions, destruction of cartilage at the site of pannus attachment and cellular reaction in the subchondral bone are also common to both diseases. The radiological appearances of affected joints, showing general decalcification, slight irregularity of articular contours and sometimes periosteal bone reaction, are also comparable. Gross destruction of bone is found in neither. It is doubtful, however, how much further this analogy may be carried, for the fundamental difference remains that we know the cause of the swine arthritis whereas we are still ignorant of the cause of human rheumatoid arthritis. In swine under the conditions of our experiments and probably also in the naturally occurring disease, the arthritis results from localisation in the joints of micro-organisms brought by the blood stream, and joint cultures at certain phases of the disease are not infrequently positive. In human arthritis, however, except for the instances of specific arthritis, bacteria of aetiological significance are not recoverable from the joints.

Our experiments seem to show the possibility of inflammatory tissue changes persisting in certain joints after the disappearance

of the infecting organism (p. 344). Unless there is some still unidentified virus or micro-organism present in the joints throughout the chronic active phase of rheumatoid arthritis, this behaviour of joint tissues may reconcile the chronicity of the disease with the sterility of the joints. It is doubtful if the joint tissues have ever been bacteriologically examined at a stage so early in the course of rheumatoid arthritis as that corresponding to the stage of bacterial invasion in *Erysipelothrix* arthritis of swine. According to this conception rheumatoid arthritis may result from the localisation of bacteria in the joints following a transient bacteraemia and these bacteria start tissue reactions which continue in a chronic form after the infecting organisms have been destroyed. Our evidence on this point is at present no more than suggestive.

In the pig we have a chronic proliferative arthritis of known infective aetiology which can be easily reproduced and we believe that further study of this disease in an animal whose anatomy and habits are more closely related to those of man than are those of most other experimental animals may contribute materially to the elucidation of the human disease.

Serological examinations of human cases of arthritis so far conducted by us seem to rule out the possibility that swine erysipelas infection is in any way responsible for human rheumatoid arthritis. These will be published later.

The recent work of Cecil, Angevine and Rothbard (1939) on experimental arthritis in rabbits produced with streptococci and other organisms bears a general resemblance to our own work both in its scope and in its results. These authors, using animals and organisms different from those employed by us, succeeded, like us, by bacteraemic dissemination, in inducing arthritis pathologically comparable with rheumatoid arthritis of man. Taken in conjunction with our own work, this tends to show that whereas rheumatoid arthritic joints may result from an initial bacteraemia it is possible that they are the non-specific sequel of a variety of infections.

STREPTOCOCCUS HÆMOLYTICUS AND SWINE ARTHRITIS

Hæmolytic streptococci were isolated from the throat flora on 8 occasions in 12 pigs. Hæmolytic streptococci (group C) were also found in the pus of an abscess of cheek in pig B and in association with *E. rhusiopathiae* in the pus of an empyema in pig A. These were thought to be unrelated to the arthritis present in these animals.

Sekiguchi and Irons (1917) and some other writers have attributed a proportion of cases of arthritis in swine to streptococcal infection. The reputed association of hæmolytic streptococci with rheumatic processes in man is well known. We therefore administered streptococci and streptococcal products by various

routes to 8 pigs but intravenous injections of living streptococci were not made. No arthritis resulted from any of these experiments and all pathological effects of the injections seemed to be local and transient. These experiments were made before we had been successful in inducing arthritis by intravenous injections of *E. rhusiopathiae* and it would be interesting to test whether hæmolytic streptococci given intravenously would set up a similar type of arthritis.

Increase of the anti-hæmolysin content of the serum followed experimental infection with hæmolytic streptococci but not the injection of filtrate. Significantly high titres (960 M.H.D. or more) were met with in the sera of certain animals (B, C, D 2, D 3, F 2 and F 4) which were known or presumed to suffer from *Erysipelothrix* arthritis. Streptococcal infection in these animals remained otherwise undetected except in pig B, in which there was a subcutaneous abscess of the cheek. All the other pigs with high anti-hæmolysin titres had been in contact with pig B and it is possible that they had contracted a streptococcal infection from this animal, though the sites of such infection were not revealed either during life or at post-mortem. Other swine with *Erysipelothrix* arthritis showed no such rise of anti-hæmolysin titre and there remains, therefore, no proof that streptococcal infection had contributed to the development of the arthritis.

VITAMIN C DEFICIENCY AND ARTHRITIS

Rinehart and Mettier (1934), Rinehart, Connor and Mettier (1934) and Rinehart (1935-36) showed that chronic scurvy with superimposed infection in the guinea-pig results in an arthropathy with tissue changes bearing a marked resemblance to those found in the rheumatic diseases in man, and though Schultz (1936) denied that the experimental lesions bore more than a slight resemblance to rheumatic tissue change, Glasunow (1937) and others have clearly shown the importance of vitamin C in the maintenance of healthy mesenchymal tissues.

We attempted to induce arthritis by depriving swine of vitamin C and to influence the course of experimental infections in others by adding excess of vitamin C to the diet, but no conclusive results were obtained. No influence upon the pathogenic effects of streptococci or *Erysipelothrix* was noted. We saw none of the phenomena associated with true scurvy and we did not attempt to assay the vitamin C content of rations, excreta or blood.

SUMMARY

1. Pathological and bacteriological studies were carried out on 9 cases of natural polyarthritis of swine. Evidence of infection with swine erysipelas was established in 4 of these animals.

2. A similar arthritis followed repeated intravenous injections of cultures of *E. rhusiopathiae* in all of 8 experimental swine.

3. Subcutaneous injections of *E. rhusiopathiae* failed to produce arthritis in 4 swine but observations on these animals were continued for only a short time.

4. Both the experimentally induced and the naturally occurring disease can be described as a chronic proliferative arthritis. Its course and the sequence of pathological events in the joints are described.

5. Arthritis in the experimental swine resulted from bacteraemia and organisms were cultivated from joint material, particularly in the early stages of the disease. Some animals harboured viable organisms up to 8 months after injection, but many grossly arthritic joints were sterile.

6. Arthritis followed single injections in 6 pigs, only 3 of which had been rendered hypersensitive to an autolysate of *E. rhusiopathiae*. This would suggest that a state of hypersensitivity is not essential for the development of the arthritis.

7. In agglutination tests against *E. rhusiopathiae*, high titres were not always found in the presence of infection.

8. In animals experimentally infected with *E. rhusiopathiae*, focal inflammatory polyarteritis, focal necrosis of liver and myocardium, lymphadenopathy, circulatory monocytosis and endocarditis were found but no skin manifestations were ever encountered.

9. Comparisons are made between swine arthritis and chronic arthritis in man.

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576.809.4:582.28 (*Erysipelothrix rhusiopathiæ*)

STUDIES ON *ERYSIPELOTHRIX RHUSIOPATHIÆ*

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OUTBREAKS of swine erysipelas are usually though not always successfully controlled by the use of immune serum. To ascertain if the occasional failures might be due to antigenic differences between strains of *E. rhusiopathiæ* a study was made of 43 strains collected from America, Japan, England and other parts of Europe. They were divided as follows.

English—Nos. 1-6, 9, 11-16, 22, 25-27, 29-35.

French—Nos. 17-21, 23.

German—Nos. 7, 8, 28, 43.

Japanese—Nos. 24, 37-39.

American—Nos. 10, 36, 40.

Dutch—Nos. 41, 42.

All had been isolated from pigs except 17, 21, 23 and 31, which were ovine strains.

GENERAL OBSERVATIONS

Cultural characteristics

All the strains studied were S forms and were kept so by passage through mice when necessary. In none could capsular substance or flagella be demonstrated by either positive or negative staining. All produced acid but no gas within 48 hours in glucose, maltose, lactose, lævulose, starch and dextrin, while some, after further incubation for 14 days, produced a slight but definite acid reaction in arabinose (6 strains), dulcitol (10 strains), glycerol (8 strains), inulin (12 strains), mannitol (8 strains), sucrose (12 strains), xylose (10 strains) and inositol (4 strains). Salicin was not fermented.

The reactions did not permit of any differentiation into groups. This general result agrees with those of Cornell and Glover (1925), who stated that their strains were biochemically similar though none fermented sucrose, mannitol, dulcitol, arabinose, xylose or glycerol (see also Deem and Williams, 1936).

Of the various media employed for the cultivation of *E. rhusiopathiæ*, the one which gave the best growth without too great a tendency to S-R variation was 1.0 per cent. glucose agar, pH 7.8.

In 1 per cent. glucose broth (pH 7.8) growth was usually abundant. In several experiments made to ascertain the point of maximum viable growth, a bottle containing 100 ml. of glucose broth was inoculated with 0.5 ml. of a 24-hour broth culture, producing an initial count of about 1×10^6 per ml. on glucose agar. Counts made after 6, 12, 15, 18, 24 and 48 hours' incubation at 37° C. showed a rise from just under 100×10^6 per ml. at 6 hours to a maximum of about 200×10^6 at 18-24 hours, followed by a rapid fall to 10×10^6 per ml. at 48 hours.

Virulence

To ascertain the suitability of the various methods of infecting small laboratory animals and to test the value of the scarification method for recovering the organism from highly contaminated sources, experiments were made with glucose broth cultures of a few selected strains. Viable counts were made and various dilutions in broth—which, as pointed out by Gajzágo (1932) and Viriden (1936), is preferable to saline as a diluent—were inoculated intravenously, intraperitoneally and by scarification into mice and intramuscularly and by scarification into pigeons. For scarification, the breast of the pigeon or the back of the mouse was scratched with five parallel lines and five at right angles covering an area approximately 1.5 cm. square. The dose of organisms was then dropped on the area and rubbed in with the finger encased in a rubber glove. Four animals were used for each dose and were observed for 14 days. The average infecting dose necessarily varied with the virulence of the strain but the comparative results were roughly similar whichever strain was used. With a strain of high virulence in pigeons, infection by intramuscular injection and by scarification occurred with the same dose of organisms (*ca.* 500), while in mice the intraperitoneal route, with an average lethal dose (A.L.D.) of 2000 organisms, was preferable to scarification or intravenous injection, in both of which a dose of some 50,000 organisms was required to cause a 50 per cent. mortality.

In view of the negative results obtained by Stryszak (1937) with most non-specific substances, the effect of mucin was tested. This was prepared and sterilised by the method advocated by Miller (1934-35) and by Nungester and Jourdonais (1936). A portion of the emulsion was also sterilised by autoclaving. In several experiments 0.6 ml. of each of a series of decimal dilutions of a highly virulent and of a weakly virulent strain was added to 2.4 ml. of mucin solution with a final relative viscosity of about 10. The mixture was then injected in doses of 0.5 ml. intraperitoneally into mice.

With a highly virulent strain there was no significant decrease in the infecting dose but with a less virulent strain the infecting

dose was reduced to between $\frac{1}{100}$ and $\frac{1}{1000}$ of that normally required to cause death. Typical results with one moderately virulent strain are shown in table I.

TABLE I

Effect of suspension in mucin on the infectivity of weakly virulent strain 6 of E. rhusiopathiæ

Dose (ml.)	Number of mice which died within 14 days after inoculation with culture		
	suspended in broth	suspended in mucin sterilised by	
		alcohol	autoclave
10^{-3}	4	4	4
10^{-4}	1	4	3
10^{-5}	0	4	1
10^{-6}	0	2	0

Four mice inoculated with each dose of each suspension

It will also be seen that, as noted by previous workers, mucin sterilised by alcohol was more effective than that sterilised by autoclaving.

AGGLUTINATION TESTS

Preparation of antisera

Many types of antigen prepared as detailed on p. 358 were injected intravenously into rabbits in various doses and at varying time intervals. The method yielding the most satisfactory results was the injection of four doses of 25×10^8 , 40×10^8 , 60×10^8 and 60×10^8 organisms at four day intervals. Ten days after the final injection the animals were bled out if the agglutination titre of their serum was found to be about 1:1600-1:3200. Any animal which failed to give such a response was discarded. Higher titres were not required, as it was considered that they would tend to obscure any slight differences between the strains by causing greater cross agglutination, as recently discussed by Miles (1939).

Agglutination tests with living organisms

A series of sterile dilutions of the sera was made in 1 per cent glucose broth (pH 7.8) in ordinary test tubes each containing 4.5 ml. A fixed amount (2 drops) of a 24 hour glucose broth culture was then added to each tube and the series incubated overnight at 37°C. The lower dilutions of serum caused clumping of the bacteria, the supernatant liquid remaining clear. As the dilutions increased a tube was reached where the supernatant liquid became turbid, the dilution of serum in this tube was read as the titre of the serum.

It was found that the strains could be divided into two groups not very sharply demarcated one from the other. The large

majority fell into one group and only five into the other. Each group agglutinated to a fairly high dilution with sera against organisms from the other. Representative results are recorded in table II.

TABLE II.
Results of agglutination tests with growing organisms

Serum prepared against strain	Lowest dilution of serum in which growth with turbidity occurred								
	Tested with strain no.								
	6	13	32	36	2	10	24	38	39
32	1000	1000	1000	1000	200	100	100	200	100
10	200	200	100	200	800	1000	800	1000	1000

The serum prepared against strain 32 clumped strains 6, 32 and 36 to a much higher dilution than strains 2, 10, 24, 38 and 39, while the reverse was true of the serum against strain 10. All the other strains tested fell into the group in which the higher agglutination occurred with serum 32. The division of the strains thus indicated was further examined by agglutination and passive protection experiments.

Direct agglutination tests

Various methods were investigated for the preparation of suitable antigen. Four of these may be mentioned.

(1) *Cultures on solid media.* The organisms were grown on 1 per cent. glucose agar in Roux flasks for 48 hours at 37° C., washed off in 1 per cent. formal-saline and allowed to stand for three days. The suspension was then repeatedly shaken with beads and used as antigen. This product was not of much value as it was difficult to obtain stability, the suspension was rarely homogeneous and non-specific clumping occurred.

(2) *Whole cultures on liquid media.* Cultures grown in 1 per cent. glucose broth for 48 hours were used as antigen directly after being killed by heat (56° C. for 30 minutes) or by formalin (0.5 per cent.). This method was not entirely successful as the cultures were often of insufficient density to yield easily readable results when mixed with equal quantities of serum dilutions. Some tests were carried out using 9 parts of antigen to 1 of serum but the readings obtained were rarely as definite as those obtained with more concentrated antigens.

(3) *The method of Schoening and Creech (1933).* In this method broth cultures are centrifuged and the deposit is shaken with beads in 1 per cent. formal-saline and filtered through cotton wool. Fairly good results were obtained but the method was laborious and a considerable amount of antigen was lost in filtration.

(4) *The method adopted.* Approximately 2 ml. of a 24-hour broth culture were inoculated into 2 l. of freshly prepared 1 per cent. glucose meat infusion broth at pH 7.8. After 48 hours' incubation at 37° C. the cultures were centrifuged and the deposit re-suspended in distilled water containing

1:2000 merthiolate so as to form a milky suspension. This stock suspension was usually sterile within three days and when required for use was diluted with distilled water to the opacity of Brown's tube no. 2 ($=600 \times 10^4$ *Bact. coli* per ml). As it is difficult to standardise suspensions of this organism in the small tubes commonly used for the purpose, a standard was made up in a 100 ml. bottle and suspensions for use compared with it. Distilled water was used, as the suspensions remained stable longer in the absence of salt. Moreover, as the serum dilutions were made in 0.9 per cent. saline, the salt concentration of the serum antigen mixtures was 0.45 per cent., the optimal concentration for agglutination with most of the strains.

Sera prepared by the intravenous injection of antigens of this type into rabbits were used in agglutination tests. The results were read after 4 hours' incubation in a water bath at 42°C , and again after a further 18 hours' incubation at 37°C . There was rarely any increase in titre after the reading at 4 hours.

In table III (p. 360) the results of agglutination tests with two sera and various strains are shown. Here again the strains can be divided into the same two groups, a smaller of five strains and a larger comprising all the others. The division is not absolute, since considerable cross agglutination occurs.

In order to determine if there was any fixed ratio in the proportions of these antigens, agglutination tests were made with the optimal proportions technique as utilised by Duncan (1932) for the analysis of the antigens of *Br. abortus* but failed to reveal any constant optimal proportions or to yield any information of value for the differentiation of the strains into groups.

Agglutinin absorption tests

The technique followed the usual lines. The sera were diluted 1:50 with saline and added to equal volumes of antigen suspensions of a strength equivalent to $10,000 \times 10^4$ *Bact. coli* per ml. The mixtures were allowed to stand for 24 hours at 37°C , centrifuged and the deposit discarded. The centrifuged deposit from a similar volume of fresh antigen was then added to each of the supernatants and the tubes were well shaken and replaced in the incubator. This procedure was repeated until no further agglutination occurred, or at the least, three times.

The results with two sera are shown in table IV and are sufficient to demonstrate that the two groups indicated by the direct agglutination tests possess different antigens. Since the specific titres of the absorbed sera were low it was thought that the specific antigens were present only in small amount.

Agglutination and agglutinin absorption tests with heat stable antigens

It was found that if an antigen was washed free of merthiolate by three washings with distilled water and re-suspended in distilled water it remained an excellent antigen after heating in a steamer to 100°C for 4 hours. Occasionally flocculation occurred but,

on shaking, the suspension dispersed and did not auto-agglutinate. On the other hand, if heating preceded treatment with merthiolate, the suspension frequently proved unstable.

TABLE V

Results of direct agglutination tests with heated antigens

Serum	Prepared against heated antigen of strain	Titre against heated antigen of strain									
		32	33	36	40	37	24	10	2	38	39
208	24	—	—	—	—	—	1600	800	800	800	800
297	36	3200	1600	3200	3200	3200	—	—	—	—	—

— = no agglutination in dilutions < 1 : 100.

Sera were prepared from these heated antigens and the usual agglutination tests performed. These (table V) again revealed the two groups. In this case, however, no cross agglutination occurred, even though the titre with the homologous antigen was fairly high. As shown in table VI absorption with the heterologous strains failed to remove and in some cases to reduce the agglutinins for the homologous strains.

It is therefore possible to divide strains of *E. rhusiopathiæ* into two groups in which the relative proportions of heat-stable and heat-labile antigens vary. In order to ascertain if these differences had any immunological significance passive and active immunisation experiments were carried out in mice and pigeons. For these comparative experiments it was necessary to know beforehand the approximate lethal dose. The average lethal dose (A.L.D.) of a glucose broth culture—the dose required to kill 50 per cent. of the animals inoculated—varied considerably from culture to culture of any given strain. This difficulty was avoided by re-suspending centrifuged cultures in horse serum and drying *in vacuo* by the method of Elser, Thomas and Steffen (1935).

The dried suspensions were stored at 4° C. and were tested each week by emulsifying the contents of three tubes, to obviate sampling errors, in 30 ml. of sterile tap water. This was termed a 1 : 10 dilution and serial decimal dilutions were made therefrom. A dose of 0.5 ml. of each dilution was then injected intraperitoneally into each of four mice. During the first six weeks of storage the A.L.D. increased rapidly, but after about the sixth week it remained relatively constant. Suspensions with a relatively steady killing dose were thus obtained from dried cultures stored for at least six weeks.

The results of tests on two representative strains are shown in table VII. It will be seen that with strain 24 the A.L.D. increased from $\frac{1}{20000}$ ml. after one week's storage to $\frac{1}{2000}$ ml. after 6 weeks.

TABLE VI
Agglutinin absorption tests using heated antigens and their corresponding sera

Serum	Prepared against heated antigen of strain	Titre	Absorbed with strain	Titre (after absorption) against strain									
				32	33	36	40	37	24	10	2	38	39
208	24	1600	32	—	—	—	—	—	400	200	200	400	800
			33	—	—	—	—	—	400	400	400	200	400
			36	—	—	—	—	—	400	200	400	400	800
			40	—	—	—	—	—	400	200	800	400	800
			37	—	—	—	—	—	200	200	400	200	400
			24	—	—	—	—	—	—	—	—	—	—
			10	—	—	—	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—	—	—	—
			38	—	—	—	—	—	—	—	—	—	—
			39	—	—	—	—	—	—	—	—	—	—
297	36	3200	32	—	—	—	—	—	—	—	—	—	—
			33	—	—	—	—	—	—	—	—	—	—
			36	—	—	—	—	—	—	—	—	—	—
			40	—	—	—	—	—	—	—	—	—	—
			37	—	—	—	—	—	—	—	—	—	—
			24	800	800	1600	1600	800	—	—	—	—	—
			10	1600	1600	1600	1600	1600	—	—	—	—	—
			2	800	400	1600	800	800	—	—	—	—	—
			38	1600	800	1600	1600	1600	—	—	—	—	—
			39	800	800	1600	1600	1600	—	—	—	—	—

— = titre < 100.

It then remained fairly constant, only falling to $\frac{1}{2000}$ after 12 weeks. Strain 32 shows a similar result, but with a rather sharper drop in infective power to $\frac{1}{8000}$ ml. after only 3 weeks' storage, though again $\frac{1}{2000}$ ml. was the A.L.D. after 12 weeks.

TABLE VII

Showing the increase with time in the A.L.D. of bacteria dried in vacuo and stored at 4° C.

Dose of organisms (ml.)	Result of inoculation of various doses after storage for various periods									
	Strain 24					Strain 32				
	Period of storage (weeks)					Period of storage (weeks)				
	1	3	6	9	12	1	3	6	9	12
$\frac{1}{250000}$	+	~	-	..		+	-	-
$\frac{1}{25000}$	+	+	-	-		+	-	-
$\frac{1}{2500}$	+	+	-	-		+	-	-	-	...
$\frac{1}{250}$	+	+	+	+	-	+	+	+	+	-
$\frac{1}{25}$	+	+	+	+	+	+	+	+	+	+
$\frac{1}{5}$	+	+	+	+	+	+	+	+	+	+

4 mice inoculated with each dose.

+ = 2 or more mice died within 14 days.

~ = 3 or more mice survived.

This method of maintaining a reasonably fixed infecting dose was adhered to throughout the experiments, the A.L.D. of the suspensions being noted after 8 weeks' storage and the experiments carried out one week later.

PASSIVE PROTECTION TESTS IN MICE

The mice employed were all albinos of the same parent stock and were of equal age and weight. The infective doses were graded dilutions of dried bacteria as described above. Two methods were utilised; in one, the amount of serum was kept constant while the infecting dose was varied; in the other, the dose of the organism was constant and the quantity of serum varied. The former was to be preferred. In these tests 0.3 ml. of serum was injected intraperitoneally into mice and was followed 24 hours later by a series of graded doses of the various strains under examination.

In table VIII are shown the results of one such test with sera prepared against both heated and unheated antigens of strains from each group. The serum against unheated antigen of strain 32 protected 4 out of 6 mice injected with 250 lethal doses of this strain but failed to protect against more than $2\frac{1}{2}$ lethal doses of strain 24, which belonged to the other serological group. The reverse was true of the serum prepared against strain 24.

TABLE VIII

Results of passive protection tests in mice

Type of serum	Intra-peritoneal dose of serum (ml.)	Number of mice surviving after 15 days											
		Strain 32						Strain 24					
		Dose of bacteria (A.L.D.)						Dose of bacteria (A.L.D.)					
		$\frac{1}{2}$	1	2 $\frac{1}{2}$	10	50	250	$\frac{1}{2}$	1	2 $\frac{1}{2}$	10	50	250
Normal	0.5	4	0	0	0	0	0	4	0	0	0	0	0
Prepared against unheated antigen of strain 32	0.3	5	...	5	5	4	4	6	...	4	1	1	0
Prepared against unheated antigen of strain 24	0.3	6	...	6	1	1	0	6	...	5	5	5	5
Prepared against heated antigen of strain 32	0.3	6	...	2	0	0	0	6	...	3	0	0	0
Prepared against heated antigen of strain 24	0.3	6	...	0	0	0	0	6	...	2	0	0	0

Four mice inoculated with each dose of bacteria in normal serum group and 6 in each immune serum group.

It appears therefore that these antigenic variations are related to the infective power of the organisms, and that the antigens involved in the protection experiments are of the heat-labile type, since sera prepared against heated antigens failed to protect mice against ultimate death though they did delay it somewhat, as shown in table IX.

TABLE IX

Delay in death of mice protected by sera prepared against heated antigens

Strain	Dose of bacteria (A.L.D.)	Average time (days) from injection till death in mice protected by		
		normal serum	serum against heated antigen of	
			strain 32	strain 24
32	2 $\frac{1}{2}$	6	11	7
	10	4	9	7
	50	4	7	7
	250	3	9	6
24	2 $\frac{1}{2}$	6	11	9
	10	5	7	7
	50	5	6	7
	250	3	5	5

ACTIVE IMMUNISATION TESTS IN PIGEONS

In view of the importance of vaccination as a means of controlling the disease in the field, it appeared desirable to determine whether

active protection against strains of one serological group could be conferred by the use of organisms of the other group

Strains from each group were grown on glucose agar in Roux flasks for 48 hours and washed off with 0.25 per cent formal saline. After standing for four days the suspensions were standardised by opacity to an equivalent of 20×10^9 *Bact coli* per ml (i.e. 5 times Brown's tube no. 10) and tested for sterility. They were then injected in doses of 1.0 ml into a number of pigeons. After 21 days the pigeons were again inoculated with 2.5 ml of vaccine. Another batch of pigeons was inoculated with formalised vaccine prepared according to the method of Muromtsev (1935, Muromtsev and Matvenko, 1936). For this vaccine the organisms are grown in semi solid agar (0.25 per cent) and killed by the addition of 0.3 per cent formalin. Two weeks after the second dose of vaccine, the pigeons were injected with graded doses of suspension prepared from organisms dried and stored *in vacuo*.

TABLE X
Vaccination of pigeons

Infecting strain	Dose of bacteria (A.L.D.)	Number of pigeons which died within 14 days		
		Unvaccinated controls	1 pigeons vaccinated with	
			formolised vaccine of strain 32 from liquid medium	formolised vaccine of strain 32 from semi solid medium
32	$\frac{1}{2}$	0	0	0
	1	4	6	1
	4	4	8	5
	20	4	7	4
	80	4	10	7
	320	4	10	10
24	$\frac{1}{2}$	0	0	
	1	2	8	
	4	4	8	
	20	4	9	
	80	4	10	

Four unvaccinated and 10 vaccinated pigeons inoculated with each infecting dose of bacteria

As will be seen in table X the vaccine from the liquid medium protected some of the pigeons against infecting doses up to 20 A.L.D. of both strains, whilst that from the semi solid medium, which was considerably weaker, being equivalent to about 500×10^6 *Bact coli* per ml (Brown's tube no. 2), protected 50 per cent against 20 and a few against 80 A.L.D. of the homologous strain. Despite the somewhat disappointing results it was decided, in view of the occasional reports of successful results following the use of vaccine in the field, to examine the effect of these vaccines directly on pigs.

ACTIVE IMMUNISATION TESTS IN SWINE

Field trials

In all, 200 pigs of three months old were divided into vaccinated and control groups. Some were reared away from any possibility of infection, others at the infected farms. All, previous to the experiment, were normal in every respect, with sera giving negative agglutination reactions in a dilution of 1 : 20. As the animals grew older the titre tended to rise but never exceeded 1 : 80. They were exposed to infection at various farms where swine erysipelas was endemic or where an outbreak was in progress. In no case did any pig, either vaccinated or control, show symptoms of swine erysipelas, and when slaughtered for food at about 7-8 months old all were free from any signs of infection. Cultures of *E. rhusiopathiae* were obtained however from the spleens of two, though their weekly temperature and agglutination charts did not reveal any evidence of the presence of disease.

It was therefore not possible under field conditions to demonstrate any protection due to vaccination. Attempts were then made to demonstrate the presence of protective antibodies in the sera of vaccinated pigs.

Passive protection tests

Four pigs (33-36) were vaccinated and three (37-39) were kept as controls. Fourteen days after the second dose of vaccine a quantity of blood was collected from the ear veins of both vaccinated and control animals and allowed to clot. The sera were then filtered through Seitz filters and injected intraperitoneally into mice in doses of 0.5 ml. Twenty-four hours later the mice were injected intraperitoneally with a culture of the strain used for preparing the vaccine. Four mice were used to each dose. The results failed to show the presence of protective antibodies in the sera.

Intradermal tests in pigs

During the course of the foregoing experiments several strains of *E. rhusiopathiae* had been tested on pigs in attempts to transmit the disease. It was found, as reported by most authors (Böhme, 1925; Creech, 1933), that in most cases though large doses were administered—up to 15 ml. of a broth culture—intravenously, intraperitoneally and subcutaneously the animals did not die, though many became ill. It was further noted that relatively small doses, e.g. one-fourth of an agar slope culture, if rubbed into the scarified skin behind the ear or injected intradermally, usually gave rise to a local reaction evidenced by a purple area 1-2 inches in diameter at the site of inoculation, together with a general reaction.

A strain was then encountered which was highly virulent for pigs. This has been maintained by pig-to pig passage or by storage of infected pig spleen in the cold room. When required for use a portion of the spleen was rubbed over the scarified breast of a pigeon, and when death occurred, usually on the second day, a culture was made on glucose agar slopes from the spleen of the pigeon and the resulting growth was emulsified in broth and used to infect pigs.

In order to ascertain if vaccination with the formolised vaccine conferred any immunity on pigs, this virulent strain was employed in the following experiment.

Twenty six pigs from three litters, of an average age of 7 months, were divided into three groups. Eight were vaccinated as before but with double the dose of vaccine, eight were vaccinated with the vaccine of Muromtsev in doses of 3.0 ml followed by 10 ml, six were left as controls and four were reserved for protection with 40 ml of hyperimmune swine erysipelas antiserum, which was injected subcutaneously 24 hours before the test dose of organisms. Fourteen days after the second dose of vaccine a non vaccinated pig was injected intradermally behind the ears with a series of graded doses of the virulent organism. It was found that approximately $\frac{1}{2}$ of an agar slope culture suspended in broth produced a minimal positive skin reaction. A similar dose was then used to inject all the pigs intradermally behind the ears.

The results (table XI) failed to demonstrate any protection from the vaccine. In all the vaccinated and unvaccinated animals a local positive reaction, *i.e.* a purple area of greater or less extent, was obtained, whereas those protected by serum showed no local reaction. Further, a large proportion of the vaccinated animals showed signs of systemic disturbance as evidenced by a rise of temperature and the appearance of purple areas on other parts of the body.

It appears, therefore, that vaccination by this method does not confer on a pig sufficient immunity to protect it against a minimal skin reacting dose.

DISCUSSION AND SUMMARY

It was not possible to separate strains of *E. rhusiopathiæ* on the basis of morphological or cultural examination. Of 43 strains studied serologically 38 appeared to be of one antigenic type and five of another. Of these five three were from Japan, one from England and one from America. There was no correlation between animal host and serological group or between group and virulence. Each group possesses a heat stable specific antigen. In addition, each probably contains two heat labile antigens which are present in different proportions in the two groups and are responsible for cross agglutination, they are also the important antigens with regard to resistance to infection. Sera of low potency containing antibodies to these antigens will protect mice against

TABLE XI

Intradermal reaction in vaccinated pigs (dose: 0.2 c.c. of broth suspension containing $\frac{1}{4}$ of a 24-hour culture on an agar slope)

Group	Pig no.	Reactions at various times after infection											
		24 hours		48 hours		72 hours		96 hours		120 hours		144 hours	
		Local reaction	Temperature	Local reaction	Temperature	Local reaction	Temperature	Local reaction	Temperature	Local reaction	Temperature	Local reaction	Temperature
Vaccinated with liquid formalised vaccine	1	++	104.0	+	102.0	-	100.5	-	100.5	-	103.0	-	100.5
	2	++	103.0	++	101.0	++	102.0	+	102.0	-	104.5	-	104.5
	3	+	103.0	-	103.0	-	101.0	-	101.0	-	101.5	-	101.5
	9	++	103.5	+	104.5	-	103.0	-	103.0	-	102.5	-	102.5
	10	++	104.5	++	103.0	++	102.5	-	102.5	-	106.0	-	102.5
	11	+	103.0	-	102.0	-	102.5	-	102.5	-	101.5	-	102.0
	12	++	102.0	++	103.0	++	105.0	++	105.0	++	102.0	++	104.5
	13	+	103.0	++	101.5	++	103.5	++	103.5	++	100.0	++	102.5
	20	++	103.5	+	103.0	++	106.0	++	106.0	++	106.0	++	104.5
	21	++	104.0	++	104.0	++	106.0	+	106.0	-	106.0	-	104.5
	22	++	103.5	++	107.0	++	Dead	++	...	++	...	++	...
	23	++	103.0	++	102.0	++	104.0	++	104.0	++	103.0	++	104.5
Vaccinated with solid formalised vaccine	24	++	102.0	++	107.0	++	107.0	++	107.0	++	107.5	++	106.5
	25	++	103.0	-	106.0	-	106.5	-	106.5	-	103.5	-	106.5
	26	++	103.0	++	106.5	++	103.5	-	106.5	-	107.0	-	104.0
	27	+	103.0	++	106.0	++	105.5	+	105.5	-	104.5	-	104.0
	4	-	102.0	-	102.0	-	102.0	-	102.0	-	104.5	-	102.5
Protected by serum	5	-	103.5	-	103.5	-	103.0	-	103.0	-	103.5	-	102.5
	30	-	103.5	-	103.0	-	104.0	-	104.0	-	103.0	-	102.5
	31	-	102.5	-	103.5	-	103.5	-	103.5	-	103.0	-	102.0
	6	+	103.5	++	102.0	+	105.5	-	105.5	-	106.5	-	102.0
Controls	7	+	103.0	+	102.0	++	102.0	-	102.0	-	103.0	-	101.5
	8	++	104.0	++	104.5	+	Dead	++	...	++	...	++	...
	10	++	103.5	++	101.5	++	101.5	++	101.5	++	106.5	++	104.5
	11	++	103.0	++	107.5	++	107.5	++	107.5	++	106.5	++	104.5
	12	++	101.5	++	103.5	++	103.0	++	103.0	++	104.0	++	106.0
	13	++	101.5	++	103.5	++	103.0	++	103.0	++	104.0	++	106.0
	14	++	101.5	++	103.5	++	103.0	++	103.0	++	104.0	++	106.0

++ = red flush over area of infection.
 + = red flush and purple centre.
 - = no reaction.
 ... = purple area up to 1 inch diameter.
 Dead = dead.

many lethal doses of an organism of the same group but not against a strain from the other group

An intradermal test was used to test active immunisation of pigs with a formalised vaccine. In the doses used this vaccine failed completely to protect the pigs against a minimal infecting dose.

I wish to thank Dr G. D. Shearer for assistance in the drying of the bacteria and Professor Dalling and Mr Bosworth of this Institute for strains of the organism. The Japanese and American strains were sent by Dr Kondo of the Ministry of Agriculture, Tokyo, and Dr Deem of Colorado State College, U.S.A., respectively, while the French strains were obtained through the courtesy of Mr Glover of the Medical Research Council Laboratories, Mill Hill, London. The experiments on pigs were made possible by a grant from the Agricultural Research Council, whose assistance is gratefully acknowledged.

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SHORT ARTICLES

616.937:615.7:547.836.3

A PHENANTHRIDINIUM COMPOUND OF MORGAN AND WALLS AS A CHEMOTHERAPEUTIC AGENT IN EXPERIMENTAL INFECTIONS WITH *T. CONGOLENSIS**

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It has been noted (Browning *et al.*, 1938) that the phenanthridinium compounds of Morgan and Walls (1938) constitute a new type of trypanocidal agents and that one of them, 7 amino 9 (p aminophenyl)-10 methyl phenanthridinium chloride, possesses curative action in mice experimentally infected with *T. congolense*. This observation may be significant practically as well as theoretically, since *T. congolense* is of chief importance among trypanosomes pathogenic for cattle in tropical Africa; further, infections caused by it are influenced only slightly or not at all by most other types of trypanocidal agents. The exceptions are antimonials—potassium antimony tartrate (Hornby, 1919) and especially antimosan (Curson, 1926; Parkin, 1930)—and the organic compound "surfen C" (Iensch, 1937; reviewed by van Rensburg, 1938), which have been much used in the field, Bayer 205 in large doses, trypanosol (Schwetz and Storek, 1930), some bismuthyl compounds (unpublished observations with R. Gulbransen), certain organic polyarsenical acids (Fournau *et al.*, 1933), synthalin (P. Browning, 1938) and undecane diamidine (King *et al.*, 1938) have also some action.

The original trials with Morgan and Walls's compound mentioned above indicated that the curative dose approached the maximum tolerated (1 mg. per 20 g. body weight injected subcutaneously). Extended work, however, showed with two strains of *T. congolense* that much smaller amounts had a distinct therapeutic action and a sixtieth to a hundredth of the tolerated dose might effect cure. Thus, after a single subcutaneous injection of the doses shown, the numbers cured out of the total treated were as follows.—with strain I, 10000 g. 5/5; 10000 g. 8/8; 10000 g. 7/7; 10000 g. 4/6, 10000 g. 5/6; 10000 g. 3/3; 10000 g. 0/4. With infections due to strain II, which is in general less easily influenced, the results with the same series of doses respectively were:—3/3, 3/4, 3/3, 2/2, 1/4 (the two smallest doses not being used). The drug was given usually when parasites were abundant in the blood. Cure was decided after a period of examination of the blood extending as a rule over at least several months, but often over six, no very late relapses were met with. On the other hand, it was confirmed that *T. brucei* infection was cured only by a dose near the maximum, even if administered when parasites were scanty in the blood.

* Work done with the support of the Medical Research Council and during the tenure of a Muirhead Scholarship by J. V. M. R.

Prophylactic action against *T. congolense* is slight, as animals are susceptible to inoculation within 4 days after injection of a large dose of the drug, parasites appearing in the blood after the same interval as in untreated controls. After a large dose necrosis of the subcutaneous tissues does not tend to occur.

A striking observation was made in the case of a heavily infected mouse which was convulsed before it received a curative dose. Next day, although convulsions had ceased, the animal seemed to be blind, since unlike a healthy mouse it would if unhindered have walked over the edge of a table; but in the following two days sight was obviously restored, as this abnormal behaviour ceased.

As regards toxicity, in rabbits a dose of 0.005 g. per kilo. of body weight injected intravenously as a 1:400 solution has caused no illness; 0.013 g. may be fatal at once, but this dose or 0.01 g. may cause immediate illness, with or without convulsions, which is rapidly recovered from, there being apparently no later ill effects.

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612.116:599.32 (Mus)

THE BLOOD VOLUME OF THE MOUSE

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When mice are used for the titration of toxins or for toxicity tests on biological substances, it is not unusual for 0.5-1 c.c. of protein-containing fluids to be injected intravenously or intraperitoneally. It has frequently been objected that such amounts are large as compared with the blood volume of the animal; information on the blood volume and its variability in the mouse appears therefore to be of some value.

Results obtained by other observers are as follows

Author	No of animals	Weight	Blood volume in c.c per kilo	Method
Dreyer and Ray (1910 11)	19	11 9 29 35 g	57 7 (45 7 70)	Mincing and extraction of hæmoglobin
von Behring, E A *	1	10 g	61	Injection of antitoxin

* We have not seen this paper and have quoted the figures from Erlanger (1921) We are unable to determine whether the value is in g or c.c per kilo The figure which is required for most physiological purposes is the volume rather than the mass of the blood

Methods

One hundred mice (46 males, 54 females) weighing from 5 to 38 g were washed out by Welcher's method The animal was anaesthetised with a mixture of chloroform and ether and pinned out on a wax plate The chest was opened and flooded with 1 6 per cent oxalate and a 0 04 c.c heart blood sample removed with a micrometer syringe and diluted to 4 or 8 c.c as convenient with 1 500 ammonia solution Larger heart blood samples should be avoided, as dilution of blood may occur during their removal The animal was then washed out with 1 per cent sodium citrate in 0 9 per cent saline at 37° C, first through the pulmonary artery and then through the aorta, with massage of the organs and limbs until the organs were free from blood and the washings colourless The washings were collected hemolysed with saponin made up to a convenient volume, centrifuged and compared with the standard made from the heart blood sample by means of a calibrated photo electric colorimeter with a logarithmic scale Due allowance was made for the opalescence of the washings by taking two colorimetric readings, one with a green (Wratten 58) and one with a red (Wratten 29) filter The "red" reading, which is almost entirely due to opalescence was subtracted from the "green" reading and the result used for reading from the calibration graph From the relation between the colour of washings and standard the total blood volume and the blood volume per kilo were determined

This method will be the subject of a further note by Dr J W Trevan

Results

100 mixed mice (fig 1) The mean blood volume per kilo is 63 2 c.c with a variability of 12 0 per cent

46 male mice The mean blood volume per kilo is 63 5 c.c with a variability of 11 9 per cent There is a small but significant negative correlation of -0.38 ($P < 0.01$, Fisher, 1936, table V, A) between the blood volume per kilo and the body weight † Following Dreyer and Ray (1910 11) and Chisolm (1911) we have determined a logarithmic relation between the blood volume and the body weight and find the best fit to be given by $BV = 0.09 BW^{0.88}$ (fig 2), which agrees very well with the value obtained by Chisolm for 129 rats but not with that of Dreyer and Ray for a much

† The weight is the crude weight, without deduction for food in the alimentary canal

smaller number of mice.* The correlation coefficient between the logarithm of the blood volume and the logarithm of the body weight is $+0.95$.

54 female mice. The mean blood volume per kilo. is 62.0 c.c. with a variability of 13.3 per cent. There is no correlation between the blood volume per kilo. and the weight ($r = 0.03$, $P > 0.1$). In view of the finding in the males this seemed to need some explanation.

Though no mouse washed out was pregnant we had taken no trouble to make sure that our females were virgins. We thought it possible that the increase in blood volume per kilo. which is generally believed to occur in pregnancy might persist into the puerperium, in which case the larger

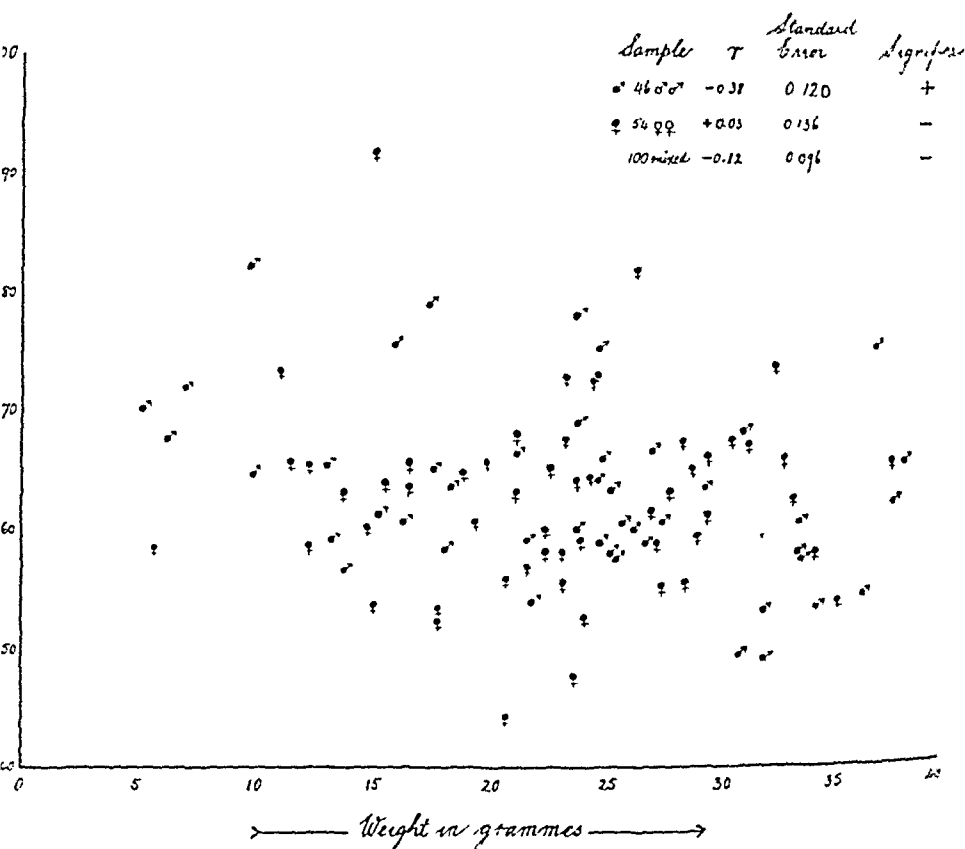


FIG. 1.—Relation between blood volume per kilo. and body weight in 100 normal mice.

females which were more likely to have had litters would show a disproportionate number with a high relative blood volume. Thus the drop in blood volume per kilo. with increasing weight which, on the analogy with males, might be expected in females would be obscured.

We therefore washed out 20 mice in various stages of pregnancy, and 10 at various times *post partum*. Great care was taken to wash out the

* Dreyer and Ray's results are on the average lower than ours; this may be due to the use of the mincing method, which we consider does not extract all the haemoglobin from the vessels. The best fit for their figures is given by $B.V. = 0.146 B.W.^{0.71}$.

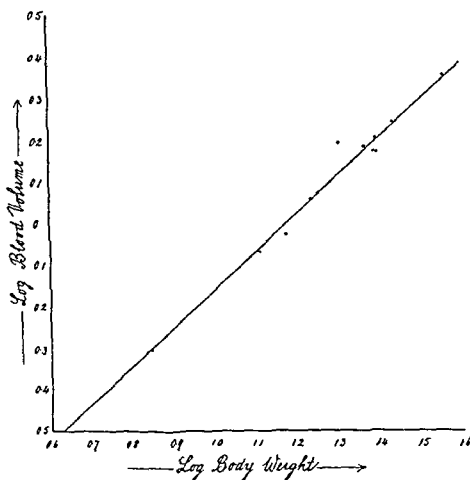


FIG 2—Relation between log blood volume and log body weight in 46 male mice
The best fit is given by $BV = 0.09 BW^{0.88}$

TABLE I—Blood volume per kilo in pregnant mice

Mother's weight including fetuses	Weight of fetuses	Blood volume in c.c. per kilo mother's weight including fetuses	Blood volume in c.c. per kilo mother's weight excluding fetuses
g	g		
31.62	0.5	71.7	72.9
31.63	1.66	59.7	63.0
31.67	0.32	65.0	65.7
31.70	< 0.05	69.1	69.1
31.74	2.57	68.3	74.8
31.83	0.55	64.1	65.2
32.05	2.59	77.1	84.1
32.99	1.93	67.3	71.5
34.81	1.25	72.1	74.8
35.00	4.9	66.0	76.7
35.86	5.3	60.0	70.3
35.96	8.95	61.5	81.8
36.3	0.51	78.0	79.1
36.6	4.79	68.3	78.6
37.16	2.92	74.8	81.1
37.3	0.1	61.4	81.2
37.96	5.62	64.8	76.1
39.11	2.3	68.6	73.5
39.11	6.91	61.9	75.1
47.6	13.23	56.0	77.4
		Mean = 67.8 c.c., not significantly greater than the mean of the 54 normal females	Mean 74.6 c.c., significantly greater than the mean of the 54 normal females

uteri, after which the foetuses with the placenta and membranes were removed and weighed. Table I shows that the blood volume per kilo. of the pregnant mice was only slightly higher than normal if the weight of the foetuses was included in the mother's weight, but was uniformly high if the foetal weight was subtracted. Fig. 3 shows that there is a fair correlation ($r = 0.51$) between the blood volume per kilo. of mother's weight less foetuses and the foetal weight; the two sets of results taken together show that the blood volume of the pregnant female mouse is roughly what would be expected if the foetuses were part of the mother's tissues.

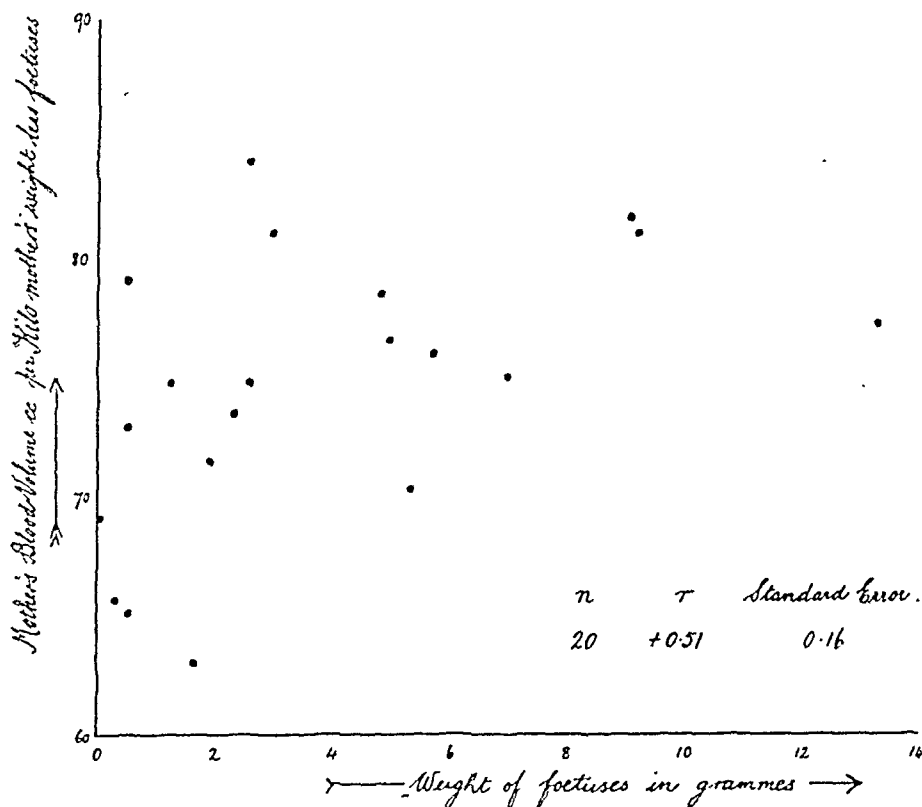


FIG. 3.—Relation between blood volume per kilo. mother's weight less foetuses and the foetal weight.

TABLE II.—Blood volume per kilo. in post-partum female mice

Mother's weight	Time post partum	Blood volume in c.c. per kilo.
30.18	<1 day	67.9
33.14	<1 "	73.3
28.75	<1 "	71.6
27.28	1 "	60.8
25.35	1 "	72.6
29.93	2 days	75.2
28.08	2 "	70.9
34.48	7 "	73.3
26.05	10-14 days	60.7
23.6	10-14 "	70.0

The blood volume of mice *post partum* shows that the increased blood volume per kilo of pregnant female mice is retained, at any rate in some animals, for as long as 10-14 days after parturition (table II). This would clearly be advantageous to the mouse, which normally goes into oestrus and becomes pregnant again immediately after parturition.

Summary

1 The average blood volume per kilo of the mouse is 63.2 c.c., with a variability of 12.9 per cent. The figures for the sexes are: 46 males, mean 63.5 c.c., with a variability of 11.9 per cent; 54 females, mean 62.9 c.c., with a variability of 13.3 per cent. The best fit for the males is given by $BV = 0.09 BW^{0.88}$.

2 There is a small negative correlation between the blood volume per kilo and the weight in male mice. In non-pregnant female mice there is no such correlation.

3 Pregnant mice have such a blood volume as would be expected if the foetuses were part of the maternal tissues, i.e. the mother provides a stroma for the foetuses.

4 The increased blood volume per kilo of the pregnant females less foetuses persists into the puerperium in some mice for at least 10 days.

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THROMBO ANGIITIS OBLITERANS IN A HORSE

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(PLATE XLII)

There appears to be no record of the occurrence of arterial disease in animals analogous to that originally described in man by Buerger (1908) as thrombo anguitis obliterans. Nieberle and Cohrs (1931) state that the disease does not occur in animals while Holz (1938) uses the term erroneously as a synonym for endarteritis obliterans in describing the vascular changes in equine infectious anaemia. Intermittent claudication is well known to occur in horses and a good description of such cases is given in Hutrya Mark and Manning (1938). In enumerating the causes of the clinical entity, these authors do not mention any condition which might be regarded as Buerger's disease. At present the commonest cause of claudication in horses is thought to be thrombosis of the abdominal aorta and its main branches secondary to invasion of the walls by the larvae of *Strongylus*.

vulgaris—a common round worm of horses. The arterial lesion in these animals is referred to in veterinary literature as “verminous aneurysm.” The possibility that horses might suffer from a condition similar to Buerger’s disease was suggested by Telford (1936), who states that “Thrombosis in the horse may be the result of larval infection of the arterial coat—of arterio-sclerosis and of embolism. These morbid processes have, however, no share in the disease of thrombo-angiitis obliterans in man. . . . There are reasons to believe that the same disease may appear in the horse and it would prove of very real interest and of vital importance to the surgeon if an equine form of thrombo-angiitis obliterans were disclosed, supported by clinical and histological details.” Incidentally it might be emphasised here that the occurrence of arterio-sclerosis in domesticated animals in the sense of atheroma (athero-sclerosis) has not been clearly established: many of the descriptions appear to be more comparable with Mönckeberg’s medial calcification of man. Our chance finding of vascular lesions simulating Buerger’s disease in a horse free from intravascular strongyle infestation is therefore of some significance.

Case report

The subject of this disease, a thoroughbred mare of 9 years, raced with success till the age of 4, when she was put to stud and not used again for work of any kind. Apart from being barren during the last four years, the mare had been perfectly well. On 20th November 1939 she received, for experimental purposes, a heavy dose of phenothiazine as an anthelmintic. She became dull and refused food the following day, seemed to recover on the 22nd, but on the 23rd was down, showed marked respiratory distress, a very fast, weak pulse and a temperature of 103° F. Later the same day, when apparently moribund, she was shot.

Summary of post-mortem findings. Pulmonary embolism; thrombosis of termination of abdominal aorta; occlusion of the large arteries of both hind limbs by thrombus and mural thickening; thrombosis and thickening of some veins in the hind limbs; numerous acute hæmorrhagic erosions in fundus of stomach; subendocardial hæmorrhages in left ventricle and right atrium; congestion of lungs; areas of mucosal necrosis and ulceration in renal pelves and ureters, probably attributable to the toxic action of the drug; no œdema of the limbs.

Vascular changes: macroscopic

A solid mass of thrombus, 20×4×3 cm., was attached to the dorsal and right lateral wall of the abdominal aorta from a point immediately posterior to the origin of the renal arteries to the terminal bifurcation. It covered the orifices of the lumbar and right external iliac arteries. Superficially it was pink, soft and easily detached, while deeper it was paler in colour and firm. Plaques of intimal thickening about 1 cm. thick, covered in part by fresh thrombus, were present at the orifices of the left external iliac and left hypogastric arteries. The hypogastric arteries were otherwise fully patent, but the external iliac arteries were occluded, the right from its origin, the left from a point 3·5 cm. from the aorta. Numerous transverse sections of the main arteries of the right hind limb revealed widespread obliterative disease extending from the external iliac to the dorsalis pedis (fig. 1). The vessels were completely occluded in segments by solid, greyish-brown tissue perforated by small openings from which could be expressed a little fluid blood. The degree of occlusion varied, and indeed occasionally the vessel was fully patent. Where occlusion was

partial, the lumen contained either recent thrombus or fluid blood. The veins were similarly affected. There was no perivascular fibrosis and no binding of arteries, veins and nerves into bundles. The heart and the mesenteric and all other arteries examined appeared to be normal.

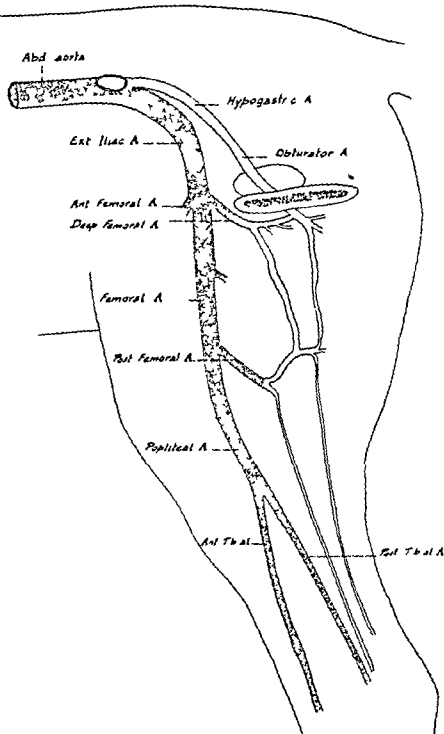


FIG. 1.—Diagrammatic representation of arteries of right hind limb. Affected vessels are shaded.

Vascular changes microscopic

The mass in the aorta was composed superficially of fresh thrombus, in the deeper layers, blood cells became progressively fewer and lay in a homogeneous, brightly eosinophilic ground substance. The base was firmly attached to the aortic wall by collagenous tissue which contained much blood pigment and a few elastic fibres (derived from the inner elastic layers) very slightly encrusted with calcium salts. The media was fibrosed and vascularised.

The lumen of the arteries of the right hind limb from the external iliac to the dorsalis pedis varied greatly in size, without regular gradation in any direction. In segments it was entirely obliterated by tissue perforated only by a few canalising vessels (figs. 2 and 3); in others it was fully patent. In these sites the vessel wall was normal. The lumen contained either blood or fresh thrombus. The obturating mass lay within a stout undulating internal elastic lamina (fig. 4). It consisted of fibrous connective tissue, partly cellular, partly collagenous, in which were capillaries and larger vessels of varying size. Around these there was sometimes slight infiltration by lymphocytes and plasma cells. Elastic fibres were scanty and almost entirely confined to the walls of the canalising vessels, which also occasionally had a few plain muscle cells in their walls (fig. 5). Blood pigment, both within macrophages and free, was fairly abundant. There was no calcification.

The media was fibrosed and perforated by vessels passing inwards from the adventitia (fig. 4). Small collections of round cells were present around some of these. No adventitial fibrosis was present. The changes in the veins were similar in nature except that the occluding fibrous tissue was more cellular (cf. figs. 4 and 6).

There was no evidence of verminous infestation in any of the vessels, and it must be emphasised that the parasitic lesion is an active endarteritis obliterans (fig. 7), often accompanied by aneurysmal dilatation.

Discussion

The vascular lesions are definitely those of organised thrombosis, and in the absence of cardiac disease and of arteritis due to *Strongylus* larvae, the assumption of primary arterial disease of the vessels of the hind limbs seems to be justified. The thrombotic process appears to have extended towards the heart and had reached the aorta at the time of death. Absence of periarteritis and periphlebitis is no contra-indication, as Turnbull (1935-36) found little or no evidence of extra-adventitial fibrosis in 17 amputated limbs from human patients with Buerger's disease. Thrombosis of the veins in this horse was probably secondary to slowing of the blood flow consequent upon the arterial obstruction. The lesions in the arteries and veins therefore conform very closely to those of thrombo-angiitis obliterans in man.

The sex of the animal should not be a great obstacle to the acceptance of our view regarding the nature of the lesions, as the disease, although rare, does occur in women. The results of the experimental work of Suzman, Freed and Prag (1938) suggest that follicular hormone possibly has a protective action against the development of Buerger's disease. Relative to this is the fact that the mare was barren for four years before death.

During life the mare showed no symptoms of lameness, but this is readily understood as she had not been galloped or worked since the age of four. Considering the degree of arterial obstruction observed at autopsy, symptoms of intermittent claudication must inevitably have developed had the animal been severely exerted. In view of the great muscular bulk of the horse's hind limbs it is of interest to note how efficient was the collateral circulation provided by the much smaller vascular bed of the hypogastric arteries.

The arterial changes, because of their chronicity, cannot be attributed to the action of phenothiazine, although the drug in the dose given may have been toxic and may have precipitated the final illness.

This type of arterial disease must therefore be added to the recognised causes of intermittent claudication of horses.

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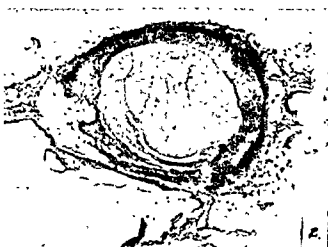
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PLATE XLII

- FIG. 2.—Right femoral artery showing organised and canalised thrombus filling the lumen. Orcein. $\times 5$.
- FIG. 3.—Right medial dorsal metatarsal vein at two levels. Orcein. $\times 5$.
- FIG. 4.—Right external iliac artery, showing vascularisation and fibrosis of media (a), distinct internal elastic lamina (b), and obturating fibrous tissue (c) containing small vessels and granules of hæmosiderin. H. and E. $\times 50$.
- FIG. 5.—Right femoral artery. The canalising vessels here have elastic and muscle fibres in their walls. Orcein. $\times 50$.
- FIG. 6.—Right medial dorsal metatarsal vein. Vascularisation of the media is very clear. The fibrous tissue within the vein is more cellular than that in the arteries and contains a vessel with fresh thrombus. H. and E. $\times 100$.
- FIG. 7.—Obturating tissue in cranial mesenteric artery from a case of "verminous aneurysm." The larval round worm is surrounded by partly necrotic inflammatory tissue in which eosinophilic polymorphs are numerous. H. and E. $\times 50$.

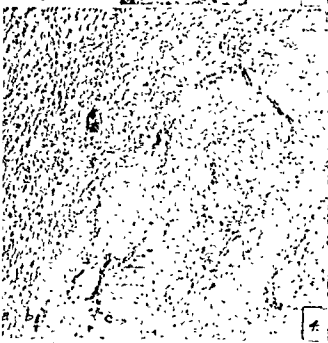
THROMBO-ANGITIS OBLITERANS IN A HORSE



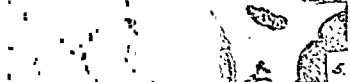
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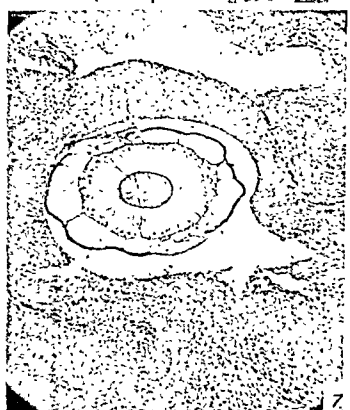
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Summary

Obliterating arterial disease morphologically identical with thromboangitis obliterans of man was encountered in a horse. The importance of this condition, hitherto undescribed in domestic animals, as a possible cause of equine intermittent claudication is indicated.

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A CONTROLLABLE MODIFICATION OF MALLORY'S
TRICHROMIC STAINING METHOD

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The original method of trichromic staining invented by Mallory (1900 01 1938) has been of immense value to all histologists but in many hands it fails to give consistent results. This may explain the endless modifications which have since been published. Of these the best known and most generally useful are the modifications suggested by Heidenhain and Masson, but they lack one of the advantages of the original method, in that fibrin and blood do not take sufficiently different colours. This distinction was of sufficient importance in our work to justify an attempt to regularise the behaviour of the original method, and as a considerable amount of our material was fixed in formol sublimate or formol saline, it meant that we started without the advantage of chrome fixed tissue. The various methods of post chroming have not proved satisfactory in our experience. The first clue to an improvement was when Dr J L Craik then working in this laboratory, noted that in a section containing picric acid, the blood failed to take acid fuchsin. By using this deviating action of the picric acid it was found possible to stain cytoplasm and fibrin in shades of red and yet leave the blood free of fuchsin. As now described, the original rather compact method of Mallory has been disarticulated, but no apology is made for this, since in the extended form the various stages are more under control and the worker of even small experience is able to produce a preparation with the colours in the right place, a fact of at least occasional value for

demonstration purposes or for photography in monotone or colour. A further advantage is the clarity of the finished result.

The first essential is a nuclear stain in shades of dark red ; this is achieved constantly by the use of celestin blue (Lendrum, 1935) and hæmalum, which in sequence give a very satisfactory blue-black nuclear stain and one which for two years has been in routine use here for van Gieson staining and for Masson's trichromic method ; the combination gives an image which is more resistant to picric acid than iron alum hæmatoxylin, and which in these methods generally needs no differentiation. In the present method the nuclear stain is firmly treated with acid alcohol and the fresher the celestin blue solution the more firmly must this be done ; although the nuclear staining may appear to be entirely removed, a mordanting effect is left in the chromatin which is not removed by picric acid and which joins with the fuchsin to give the desired red nuclear stain. The colour of the blood may be fixed at any desired shade from the very thin yellow of picric acid to a robust orange by the manoeuvre of staining the section in 1 per cent. orange G in water for any time over a minute followed by differentiation to taste with saturated aqueous picric acid. Our own preference is for the shade of yellow produced by using the orange G and picric acid in one solution ; at the concentration given below there is ample staining in two minutes but no overstaining after sixteen hours. The final differentiation of colours is rather better if the section receives long immersion. After a brief rinse in water, the fuchsin solution is filtered on to the section ; this may be a simple 1 per cent. solution of acid fuchsin (magenta acid), but the fuchsin-ponceau mixture of Masson appears to give a wider range of tones in the red. To this solution is added a small quantity of sodium sulphate, a salt which we have found to be of considerable value in preventing the growth of moulds in solutions of acid dyes. Any excess of fuchsin staining can be reduced at the time by returning the section to the picric-orange solution ; by alternating between the two solutions it is possible to emphasise the staining of the more fuchsinophil structures, such as fibrin. The remainder of the method follows the principles of Masson's modification. Incidentally quite useful staining for diagnostic purposes is obtained by omitting the fuchsin-ponceau mixture, that is to say after the picric-orange solution the section is rinsed in water and transferred to the phosphomolybdic acid and thence to the blue. Mallory (1936) now prefers phosphotungstic to phosphomolybdic acid, but the improvement is not obvious to us.

When a more forceful distinction is desired between the different colours, as for photography, it has been found a considerable gain to leave the unstained dewaxed section overnight in a jar of Hollande's fixing fluid from which the acetic acid has been omitted ; this solution is more effective than saturated aqueous corrosive sublimate or saturated aqueous picric acid as suggested by Lillie (1938), or the picro-formol-sublimate solution used earlier (Lendrum, 1939). If the end result on examination in xylol should seem too dense, it may be improved by a rapid rinse in absolute alcohol containing a little picric acid. In the past a section which had stained unsatisfactorily with Mallory's, Masson's or Gallego's method, or had thereafter faded was considered beyond recall ; if, however, the section be immersed in the picric-orange solution or a plain saturated solution of picric acid in 80-100 per cent. ethyl alcohol for half to one hour, the peculiar action of the phosphomolybdic acid is removed and after a short wash in water the section can be restained. If the nuclear method given here is used there is no need to restrain the nuclei. The partially lysed blood of post-mortem material may show some irregularity of staining, as with any dye.

Basic method

Stain in celestin blue solution, 10-20 min

Rinse in water

Filter on Mayer's (1903-04) hemalum, 5-10 min

Rinse in 95 per cent ethyl alcohol

Differentiate in acid alcohol (1 per cent HCl in 95 per cent ethyl alcohol) till the red colour ceases to come away

Rinse in 95 per cent ethyl alcohol

Stain in 0.2 per cent orange G (C.I. 27) in 80 per cent ethyl alcohol saturated with picric acid, 2 min to 16 hrs

Rinse in water, $\frac{1}{2}$ 2 min

Filter on fuchsin ponceau mixture time varies with fixative, from 15 seconds to several minutes

Rinse in 1 per cent acetic acid and examine

Decolourise the connective tissue in 1 per cent phosphomolybdic acid it is not necessary to decolourise absolutely, tissue fixed in solutions without chromates are usually sufficiently treated in one or two minutes

Stain in 2 per cent soluble blue (C.I. 706) in 1 per cent acetic acid, 2-10 min, or in 2 per cent Fast Green F.C.I. (Conn, 1936) in 1 per cent acetic acid, 2-10 min

Rinse in 1 per cent acetic acid and examine if satisfactory, dehydrate rapidly, clear in xylol and mount

Notes on staining solutions

Celestin blue solution Allow 2.5 g of iron alum to dissolve overnight at room temperature in 50 c.c of distilled water to this add 0.25 g of celestin blue R (C.I. 900) and boil the mixture for three minutes filter when cool into a staining jar and add 7 c.c of glycerol It is ready for use

Mayer's (1903-04) hemalum Allow 1 g of hematoxylin, 0.2 g of sodium iodate and 50 g of powdered potassium alum to dissolve overnight at room temperature in 1 litre of distilled water to this add 50 g of chloral hydrate and 1 g of citric acid and boil for five minutes When cool it is ready for use If not needed at once, this last boiling may be omitted The solution ripens at room temperature to an optimum at about three months, but a proportionate time of boiling will make it ready for use For example, after six weeks ripening, 2½ minutes boiling will bring the staining qualities up to maximum

The acid fuchsin used was acid magenta prepared according to the method of Scanlan, French and Holmes (1927), by the Revector Company The fuchsin ponceau mixture was made by mixing one part of 1 per cent acid magenta in 1 per cent acetic acid with two parts of 1 per cent ponceau 2R (C.I. 79) in 1 per cent acetic acid, and then adding 2.5 c.c of 10 per cent sodium sulphate to each 100 c.c of the dye solution

We had no intention of making this a rapid method, if such is desired in a trichrome stain Goldner's (1938) modification of Masson's method may be tried To those educated on it, hemalum and eosin remains the ideal routine combination, and in routine diagnostic work other methods are merely called for on occasion Their more frequent use is of considerable educative value and the method as given here makes use of solutions which, with the exception of the hemalum, can be kept for lengthy periods in staining jars or bottles We replace the celestin blue about every six months for the sake of black nuclear staining in van Gieson's and Masson's methods, the other solutions keep considerably longer

All dyes used were Revector brand

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Postscript

R. D. Lillie in a recent article (*Stain Technol.*, 1940, xv, 17) proposes Biebrich scarlet as a plasma stain in place of Masson's fuchsin-ponceau mixture. A saturated solution of Biebrich scarlet (C.I. 280) in 0.5 per cent. aqueous sodium sulphate, some two months old, was on our bench. Seventy c.c. of this was diluted with water to 100 c.c. and one c.c. of glacial acetic added; the resulting stain was used in the method given above and was found to be rapid in action and to give a pleasant colour in the final result. Although there is in general less variety of shades than with the fuchsin-ponceau mixture, this solution gives clean-cut results.

BOOKS RECEIVED

Studies on the changing incidence of peptic ulcer of the stomach and duodenum

By GUNNAR ALSTED Copenhagen, Ejnar Munksgaard London, Humphrey Milford (Oxford University Press) 1939 Pp xi and 148, 12 text figs 10s

This little book is a translation from the Danish with some alterations and additions, of various papers on the incidence of peptic ulcer contributed by the author during 1938 to the *Nordisk Medicinsk Tidsskrift* and *Hospitalstidende*. Dr Alsted's chief, Professor Meulengracht, contributes a brief foreword.

Chapter I gives a short historical account of the incidence, in so far as it is known of gastric and duodenal ulcer. The comparative merits—perhaps it would be better to say demerits—of the morbid anatomical and clinical methods of investigating this problem are discussed, and it is concluded that neither is likely to give any certain information as to the absolute frequency of peptic ulceration in the population. There can be little doubt, on the other hand that by contrast with the present day, duodenal ulcer was formerly an affliction of very rare occurrence.

In chapter II it is shown that during the last 50 or 60 years a notable change has taken place in the sex incidence of peptic ulcer. In the earlier part of this period the male to female ratio was 1.5 or 1.6. By the beginning of the century it had changed to 1.1 and in later statistics (1920-30) to 3.1. It is suggested that this change in sex incidence may be due to the increased frequency of duodenal ulcer.

Chapter III is concerned with the frequency and significance of gastric hæmorrhage. It is concluded that manifest gastric hæmorrhage is caused by ulcer in from 80 to 85 per cent of cases. This high figure is attained by lumping together cases of ulcer and cases of "gastritis" and "gastrostaxis". No attempt is made to assess the relative importance of chronic and acute ulcer in the causation of gross hæmorrhage but it would seem to be the belief of the author that even in chronic ulcer the usual source of the hæmorrhage is not the ulcer crater but "the eroded mucous membrane changed by the gastritis". We doubt if many morbid anatomists will subscribe to this view. Severe and fatal hæmorrhage in chronic ulcer is in our experience almost invariably due to the erosion of a vessel of considerable size in the ulcer floor.

In chapter IV it is shown that there has been a change in the sex incidence of manifest "gastric" (i.e. gastro-duodenal) hæmorrhage during the past 50 years similar to the change in the sex incidence of peptic ulcer itself. This is held to support the view expressed in the previous chapter as to the diagnostic importance of "gastric" hæmorrhage.

In chapter V the author shows, from a study of the annual returns of the "public medical hospital departments" in Copenhagen for the years 1876-1935, that manifest gastric hæmorrhage has been steadily

increasing, except for a brief fall during the years about 1917. The increase has been greater in men than in women and it has been most pronounced during the past 10-15 years.

In chapter VI an attempt is made to ascertain whether the increased frequency of "gastric" hæmorrhage is due exclusively to an increased bleeding tendency in cases of ulcer or whether it merely denotes increasing ulcer incidence. For this purpose all cases of affections of the "ulcer group"—i.e. practically all dyspeptic conditions except cancer, achlorhydric gastritis, cholelithiasis and a few other affections—admitted to Copenhagen hospitals during 1911-35 were added together. It was found that there was a slight fall in the frequency of these affections during the years 1911-16, after which a gradual rise set in—for males only, while in women the frequency remained unchanged. Manifest hæmorrhage showed a still greater increase in men during this period (from 1917-18 onwards) and a total increase in women. It would appear therefore that there had been increased bleeding frequency in the group of cases under review, although the increased frequency of hæmorrhage in males is no doubt partly accounted for by the increased incidence of diseases of the ulcer group.

In chapter VII all cases of peptic ulcer admitted to the Medical Department B of the Bispebjerg Hospital during the years 1914, 1919 and 1935 are passed in review. The author concludes that the apparent increase in frequency of peptic ulceration shown in 1935 is due in part to improved diagnostic technique, in part to an increase in the number of re-admissions. Corresponding with this latter observation it appears that a larger proportion of patients than before give ulcer histories of long duration, while the age incidence is moving in the direction of the older groups. Chapter VIII is a study of cases of manifest hæmorrhage occurring in the same series of patients. Unfortunately the numbers here are often very few and no attempt is made to assess the statistical significance of the differences shown. This is certainly necessary both here and in chapter VII. The conclusions arrived at, not all of which carry conviction, are that the frequency of bleeding in peptic ulcer fell from 1914 to 1919 and then rose considerably from 1919 to 1935, while at the same time the hæmorrhages became less severe and were much less often lethal.

The observations recorded in the previous chapters are reviewed in chapter IX and various generalisations made. It is concluded that the supposed increase in the frequency of ulcer is exclusively due to improved diagnostic technique and a growing number of re-admissions. The frequency of fresh ulcers is unchanged for *men* in the course of time. Ulcer now seems to be more chronic than hitherto and to be "principally localised round the pylorus and in the duodenum." It would seem that "the clinical manifestation of gastric ulcer has changed considerably in the course of the last 50-60 years, so that, while it was previously an acute disease, it is now predominantly chronic, and while previously localised principally to the body of the stomach, its seat is now the duodenum." For *women* the effect of this development is that there is now a great decrease in the frequency of ulcer, and it is believed that this is the real cause of the change in the sex ratio.

In view of the fact that the increasing incidence of peptic ulcer was temporarily checked during the war years, it is conjectured that nutritional factors may have been of ætiological importance. In this connection reference is also made to the great change which has taken place in social and economic conditions during the past 50 or 60 years.

This is a stimulating and provocative book. Here is much food for reflection and a stimulus to the more detailed study of the morbid anatomy of peptic ulcer, in particular the comparative frequency and distribution of acute and chronic ulcer of the stomach. There is a bibliography of over 100 references.

An introduction to medical mycology.

By GEORGE M. LEWIS and MARY E. HOPPER Chicago · The Year Book Publishers, Inc 1939 Pp viii and 315 ; 71 plates 27s 6d.

This is a publication in the production of which no expense has been spared to impress the uncritical reader. It is beautifully printed on heavy art paper and is illustrated with 71 plates containing 383 photographs and photomicrographs, most of them original.

The book is designed principally for post-graduate students in dermatology and for physicians practising dermatology. It is divided into two parts, of which the first deals with the clinical, theoretical and experimental aspects of human mycotic diseases, while the second describes the laboratory methods which the authors consider to be of value in the elucidation of a suspected mycosis and includes descriptive summaries of the characters and habits of the fungi listed in part I.

The authors stress throughout the importance of the contributions of American workers and there are few references to the equally valuable contributions of the modern French, Italian and German mycologists. Little information of value to the laboratory worker is contained in the first part of the book. The chapter on the structure of fungi, which occupies only one-third of a page, could be deleted without loss, even if the subject were not dealt with in greater detail in the second part. The reason for the inclusion of this chapter and of several other similar chapters in both parts of the book is obscure. The statement in chap V that improvement of the disorder after fungicidal therapy is evidence that the disease is mycotic is open to challenge—a therapeutic diagnosis is often dangerous. Chap VI is almost exclusively devoted to a review of the literature of the trichophytin test and a description of the author's work with it. The amount of space allocated to this test is out of all proportion to its value in a book purporting to be an introduction to medical mycology: all the relevant data could be adequately presented in a single page. Three pages alone are wasted on tables that could be effectively summarised in a few lines of text. The 145 pages comprising chap. IX and X contain a clearly written, readable, general account of the superficial and deep mycoses of man. *Tinea capitis*—including *favus*, *t. barbae*, *glabrosa* and *cruris*, *dermatophytosis*, *moniliasis* and *t. versicolor* in the superficial group, are considered in some detail from the clinical standpoint, and shorter descriptions of *erythrasma*, *t. imbricata*, *otomycosis*, *leptothrix* and *chromoblastomycosis* are appended. The statement that patients with any form of *moniliasis* should be considered potentially diabetic is not justifiable. The statement that a mycotic disorder may be suspected in a case of pulmonary disease when repeated tests for *M. tuberculosis* have been fruitless is also not acceptable. It is, however, encouraging to read in an American publication that the finding of monilia in the sputum in cases of suspected bronchial moniliasis is not sufficient to establish a diagnosis. It is also a relief to find that bronchomoniliasis is not included among the deep mycoses. These comprise *actinomycosis*, *blastomycosis*, *coccidiomycosis*, *granuloma paracoccidioides*, *torulosis* and *aspergillosis*. No reference is

made to Naeslund's work in the section on actinomycosis, and the work of de Magalhaes on *Oidium braziliense* is not mentioned in the section on granuloma paracoccidioides. These two chapters are profusely illustrated. The photographs of tinca and sycosis barbae, t. glabrosa, t. cruris, t. versicolor and chromoblastomycosis are good and informative. There is, however, an unnecessary multiplication of illustrations, some of which are indifferent and of no value. The frontispiece, showing a cabinet for the display of cultural material, is quite unnecessary, and it is doubtful whether any dermatologist will derive any information from fig. F, pl. 4, which shows a bald head with the explanatory legend—"complete epilation of scalp hair following roentgen treatment." These are only two examples out of many.

The second part of the book opens with two chapters, each half-a-page in length, on precautions against laboratory infections and on the microscope respectively. The information supplied is of an elementary nature, a criticism that applies with equal force to the next four chapters. The technique of the passive transfer test (chap. XXVII—half-a-page), filtered ultra-violet radiation (chap. XXIX), the trichophytin test (chap. XXX) and the oidiomycin test (chap. XXXI—one-third of a page) are not laboratory procedures. Even if these chapters were removed to part I, they are—with the possible exception of chap. XXIX—too general and too brief to be of much value to the clinician. The only matter in this part of the book that is of any value to the British laboratory worker is contained in chaps. XXXII and XXXIII (51 pp., 137 figs.), and consists of summaries of the characters and habits of the fungi mentioned in part I. Even here the bulk of the text is concerned with clinical and not laboratory data. Many of the illustrations in the second part of the book are valueless. Pl. 46, for example, contains indifferent photomicrographs of cotton, wool, plant and wood fibres—the last with an air-bubble included; crystals of KOH dried on a cover-glass, amorphous debris from lesions treated with zinc ointment, and oil globules. Other examples are figs. B and G, pl. 59; fig. B, pl. 62; fig. D, pl. 65; fig. B, pl. 66, which must surely be the worst photograph of pus ever printed; fig. C, pl. 67; pl. 69, and figs. B and F, pl. 71. Moreover, several illustrations have been duplicated quite unnecessarily. An example is that of the photograph of a mount from a young colony of *Hormodendron*, fig. B, pl. 62, which is repeated in fig. F, pl. 71. Incidentally, the only errors observed in the printing are the faulty spellings of *Hormodendron* as *Hormodendrum*, or as *Hormodencrum* in the legend of pl. 62.

The chapters on the mycoses in the first part of this book will be of value to the dermatologist and will provide the general practitioner who is interested in dermatology with a readable general account of those diseases; but the information supplied is insufficient to justify the purchase of the book by the laboratory worker. The authors admit that the bibliography is not complete, but they have compiled a useful if one-sided list of references to important publications, most of them in English.

Human histology.

By EUGENIA R. A. COOPER. London: H. K. Lewis & Co., Ltd. 1939. Pp. xiv and 423; 237 text figs. 16s.

Few will dispute the claim, clearly stated by Wood Jones in his recently published lectures on "Life and living," that human histology

should be taught as a branch of anatomy. Such too is the standpoint of the author of this book and the anatomical approach is justified by the treatment of the matter. The preliminary paragraphs in each section form a bridge between gross anatomy and histology and the choice of human tissues for demonstration wherever possible is so obviously correct that we may wonder why it is not universal. The medical student will find this a clear and concise introduction to the subject. Some account of the carotid body should have been included inasmuch as this structure is sometimes the site of tumour formation, but otherwise all essentials are to be found. Of special merit are the sections dealing with the histology of bone, in which the influence of Professor S. L. Baker's work is acknowledged, and with the endocrine and nervous systems of which Dr Cooper has special knowledge. Exception may however be taken to the statement (p. 88) that the significance of the microglial cells is unknown. It is surely well established that, whatever their origin, they are the scavengers of the central nervous system. Again it would have been helpful to the student to have clarified the character of the subarachnoid space over the spinal cord, where there truly is a space, in contrast to the sponge-like character of the pia arachnoid over the brain. An unconventional line is taken in the use of the term epithelium, the origin of which (p. 21) is postulated from ectoderm, mesoderm or endoderm. Hence the statement on p. 22 that "pavement epithelium constitutes the lining of blood and lymph vessels, of the heart and of serous membranes." Nevertheless the term endothelium is retained for the lining of vessels though not for the other structures named. This interchangeability of the terms "pavement epithelium" and "endothelium" is likely to prove perplexing to students.

The illustrations are almost exclusively microphotographs and are for the most part clear, some are excellent. In certain instances however the magnification is inadequate, the cytological details of the pineal body being particularly disappointing. While paying tribute to the scientific propriety of using microphotographs wherever possible, it is difficult to avoid the conclusion that simple line drawings are often better for teaching purposes. Thus the time-honoured diagram of the nephron, which finds no place here, has a value which makes it irreplaceable.

We cordially commend this book to the student of medicine. He will find here a clear, didactic presentation of the subject of human histology fully adequate to his needs.

Lehrbuch der Mikrobiologie und Immunbiologie

By MAX GUNDEL and WALTER SCHURMANN. Berlin: Julius Springer 1939. Pp. viii and 456, 85 text figs (53 in colour). R. M. 22 50 (bound 24 60).

This book, which also serves as a second edition to E. Gotschlich and W. Schürmann's *Leitfaden der Mikroparasitologie und Serologie*, corresponds in scope with the text book of bacteriology of moderate length published in this country and America. It follows the practice of including a survey of the pathogenic fungi and protozoa but differs from most text books of bacteriology in also containing a short chapter on the parasitic worms.

An attempt has been made to systematise the description of the viruses into those responsible for (1) general infections such as yellow

fever, (ii) infections of the skin, (iii) exanthemata, (iv) inflammations of the encephalon, (v) infections of the glandular systems, (vi) infections of the cornea, (vii) infections of the respiratory system and lastly virus-like organisms—rickettsia.

The book follows pretty closely the generally accepted teaching on bacteriology of the present day. The typhoid-paratyphoid-food-poisoning group of bacteria is very fully discussed. The more recent work on antigenic analysis is given in considerable detail but as in many text-books there is lack of such precise directions with regard to the application of these methods as would enable the uninitiated to proceed to their execution. There is for example no detailed description of the method of preparing O bacillary suspensions with a view to injection for the production of antibody and the description of the method of absorption of agglutinins is in general rather than specific terms. In fact one is left with the impression that neither the authors nor those with whom they are associated make practical use of these methods and that they depend rather on biochemical and cultural tests for a further differentiation than can be obtained with the use of a limited number of sera. For example for the differentiation of *B. paratyphosus* "B" and *B. typhi murium* (Aertrycke) special emphasis is put on the fermentation of rhamnose and xylose, the phenomenon of "Schleimwallbildung" and the reaction of the white mouse to consumption of fodder contaminated with the bacillus concerned. Death, it is stated, should follow such feeding in 5-8 days if Aertrycke or Gaertner bacilli are fed. "Schleimwallbildung" is a phenomenon specially studied by R. Müller (*Zbl. Bakt., Abt. I, Orig.*, 1925, xev, 147) and consists of the development in isolated colonies of a raised mucoid peripheral ring sharply demarcated from the central portion. It is best seen in well isolated colonies of cultures incubated at 37° C. for 24 hours and then kept for 24 hours or longer at room temperature. It is shown by colonies of Gaertner and paratyphoid "C" bacilli, although not so markedly as by colonies of paratyphoid "B" bacilli.

The classification of streptococci on pages 203 and 204 into two groups with *Str. viridans* in the first and mouth, milk and bowel streptococci in the second is not one which leaves a favourable impression.

It is to be regretted that in a book published in 1939 a brief notice of the sulphanilamide drugs is confined almost entirely to the work of Domagk, ignoring the important contributions to this subject which have been made in Paris and London.

The book has been carefully edited, only a very occasional slip being detected, for example on page 289 "Carbolfurchsinlösung."

There are many excellent coloured illustrations and the general impression left is that of a sound presentation of the current teaching on the subject.

The care of a small rat colony

By ROLAND J. MAIN. London: Henry Kimpton. 1939. Pp. 101; 12 text figs. 10s.

It is unhappily a common laboratory practice to attempt the establishment of colonies of rodents without any previous knowledge or experience of their requirements. In consequence valuable money and time are often wasted. This book is designed to supply complete information with respect to rat husbandry and it is extremely well done. But apart from being an expensive book for its size, it is

apparent that the standard of cost of upkeep in America is a good deal higher than in this country and that few institutions here could afford to run colonies on such expensive lines. One of the chief problems with which the Virginia Medical College has to contend is excessive heat in summer and elaborate precautions are taken to prevent harbouring of vermin at this temperature. It is interesting to note that evenness of temperature is regarded as of first importance for successful breeding and this will be amply confirmed by anyone who has had to do with mouse or rat husbandry.

It is a very readable and informative book and it was read with much interest and appreciation by a laboratory assistant to whom it was lent. The vignettes illustrating imaginary episodes in the life of a rat are an amusing and novel feature.

Symposium on the synapse

By H. S. GASSER, J. ERLANGER, D. W. BRONK, R. LORENTE DE NÓ and A. FORBES. Springfield, Illinois and Baltimore, Maryland. C. C. Thomas. 1939. Pp. 114, 25 text figs. \$2 (\$1.50 in paper covers).

This little book, a reprint from *J. Neurophysiol.*, 1939, 11, 361-472, assembles in a convenient form the contributions of five of the United States' most distinguished neurophysiologists to the symposium on synaptic transmission held during the meeting of the American Physiological Society at Toronto in April 1939. The mechanism of the synapse is, and is likely to remain, the subject of controversy, and this controversy has become more acute as a result of the recent discovery of the possibility of a chemical synaptic mechanism.

Professor Gasser reviews his own and his pupils' work on the properties of peripheral axons, since, as he rightly points out, a study of the axon gives us at least information of the properties of the materials which come in contact at the borders of the synapse. Professor Erlanger shows that many of the properties of the synapse can be demonstrated in nerve fibres which have been partially blocked by electrical polarization. He concludes therefore that there is no need to introduce the confusing additional concept of a chemical mediation at the neuromuscular junction or the ganglionic synapse. Professor Bronk gives a very lucid account of the work of his school on the transmission of trains of impulses through sympathetic ganglia. His attitude to the subject is probably best shown by his words: "It is necessary not to think of synaptic transmission as the development of one impulse by another. It is more probable, on the contrary, that a sequence of impulses in a number of fibers produces changes in the surroundings of a cell. The properties of the cell are thereby altered, and at a certain stage in the process an impulse is discharged." Dr Lorente de Nó occupies some three fifths of the book in a review of his work on the electrophysiology of the oculomotor nuclei, which gives impressive evidence of the precision and scope of modern physiological methods. It is perhaps regrettable that, in developing his theories of transmission by electrical means, he dismisses some of the most cogent evidence of his opponents in a curt footnote. Professor Forbes ends the volume with a judicial summing up and a warning that we should beware of adopting too readily alluring schemata of nervous function.

The book is dedicated to Sir Charles Sherrington, and every neurologist can be assured that it is a notable contribution to the science for which he has done so much.

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THE HISTOLOGY OF THE RAT'S LIVER DURING THE COURSE OF CARCINOGENESIS BY BUTTER-YELLOW (*P*-DIMETHYLAMINOAZO- BENZENE)

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(PLATES XLIII-XLVII)

THE first announcement of the successful induction of cancer of the liver by the administration of an azo dye was made by Yoshida in 1932 and the histology and histogenesis of the tumours were described in detail by Sasaki and Yoshida (1935). In an extensive investigation of related chemical compounds, Kinoshita (1937) and his co-workers found that liver cancer was much more rapidly induced by *p*-dimethylaminoazobenzene than by *o*-aminoazotoluene, the dye used by Sasaki and Yoshida.

The last-named authors give an account of the sequence of changes they saw in the liver, and appear to regard the processes as starting with hyperplasia of the periportal parenchyma, which is continuously progressive until the stage of cancer is reached. On the other hand, the results of Heep (1936-37) and Fischer-Wasels (1937) suggest that degenerative and regenerative changes play an important role in the induction of cancer by *o*-aminoazotoluene. Kinoshita appears to regard the changes in the liver as primarily hyperplastic, but admits that degenerative changes are also found. The point at issue is of some importance, as it has still to be established that any carcinogenic agent has a direct stimulating effect on cell growth, and the present communication is concerned with

the results of a histological investigation of the changes taking place in the rat's liver as the result of the addition to the food of *p*-dimethylaminoazobenzene (butter-yellow).

Experimental material and methods

The dye used was obtained from British Drug Houses, Ltd., under the name of dimethyl yellow (analar). This substance is identical with butter-yellow, and to avoid confusion in relation to the reports of previous workers, the latter term will be used. It was dissolved in a concentration of 3 per cent. in olive oil, with the help of heat supplied by a water-bath. After a few days at room temperature, a small amount of dye crystallised out from this solution, so that a saturated solution of the product used has a strength of just under 3 per cent. This is mentioned because Kinoshita speaks of 5 per cent. solutions: whether this means that the solutions were added to the food while still warm or that the substance used by him was less pure it is not possible to judge.

The rats were almost full-grown at the start of the experiments. In the first experiment 100 rats were used: their main food consisted of a mixture of approximately equal parts of broken-up maize, wheat and oats, containing 20 c.c. of 3 per cent. butter-yellow per kilo. The butter-yellow solution was intimately mixed with the maize and wheat, the oats being added subsequently so that the rats, which do not eat the husks, should be less likely to avoid ingesting an effective amount of the dye. As the liver changes did not advance as rapidly as in Kinoshita's experiments, the dose was increased after eleven weeks to 30 c.c. (*i.e.* 0.9 g. butter-yellow) per kg. of food. In addition, the rats received frequent supplements of green vegetables, and water *ad lib.* Fifty-two rats were killed at intervals and 4 died during the first eight months, and as histological examination showed that the effects were now well established the administration of butter-yellow was discontinued, in order to determine how far regression of the lesions was possible. During the ninth, tenth and eleventh months 20 were killed and 4 died. From this experiment there were thus 80 rats in all available for histological investigation; the remaining 20 were lost as the result of cannibalism.

In the second experiment the rats' food consisted of unpolished rice. This change was made because the work of Ando (1938) showed that hepatic cancer developed more rapidly in rats fed on rice than in those fed on wheat. One hundred rats were used, 50 receiving 20 c.c. and 50 receiving 30 c.c. of 3 per cent. butter-yellow in olive oil per kg. of rice. These rats received green stuff and water as in the first experiment. In the first six months 37 (18 in the 20 c.c. group, 19 in the 30 c.c. group) had been killed and 2 (in the 20 c.c. group) had died. Then, owing to the outbreak of war, the experiment was cut short, and 17 further rats (11 in the 20 c.c. group, 6 in the 30 c.c. group) were killed for histological examination on the 207th day. This experiment thus yields material for histology from 56 rats.

There was but little suggestion that the change of diet modified the hepatic reaction or that the slightly larger dose had any greater effect, so for the purposes of the present study no useful purpose will be served in separating the various groups. The results which follow, therefore, are based on an analysis of the histological findings in 136 butter-yellow-treated rats. The number examined in each month is shown in table I; the relatively small numbers in the ninth and tenth months are due to the fact that at this period the animals were being kept on a normal diet to see if regression of the liver changes occurred.

One phase in the clinical history is worthy of mention. In all the experiments there was a marked increase in the water consumption of the rats during the third month of butter yellow administration, by the fifth month the water intake had regained its normal level.

TABLE I

Summary of material available for each month of treatment

Months from beginning of treatment	Number of rats examined	Number of rats with malignant liver tumours
1	3	0
1 2	8	0
2 3	11	0
3 4	15	2
4 5	17	2
5 6	12	7
6 7	27	15
7 8	19	5
8 9	2	2
9 10	3	2
10 11	19	8
Total	136	43

Butter yellow was administered to 60 guinea pigs first in the dosage used for the rats, then progressively increased, so that for the last nine months of an experiment lasting 18 months they received the relatively enormous amount of 8 g. of solid butter yellow per kg. of food (bran). At no stage of this experiment was any change observed in the liver other than incidental infections such as pseudotuberculosis.

For histological examination the tissues were fixed in 4 per cent formaldehyde saline, embedded in paraffin and stained with Harris's hemalum and aqueous eosin, and Weigert's iron hematoxylin and van Gieson's stain. In some cases Sheridan's elastic tissue stain and the Prussian blue reaction for iron were also used. The liver and spleen were examined in all cases, other organs only in the few instances in which a lesion was present. In several cases, portions of liver were fixed in absolute alcohol for the demonstration of glycogen, and frozen sections of formaldehyde fixed material were stained with sudan III for fat.

Macroscopical changes

The earliest obvious change was in the spleen. In rats killed after four weeks or longer it was considerably enlarged, firm and rounded, and dark purplish red in colour. It attained its maximum size during the third and fourth months of treatment, when the weight ranged from 2.0 to 3.1 g. (average of 18 normals = 1.1 ± 0.08 g.), thereafter it was less enlarged and in the latest phases might even be reduced in size. In the early phase of enlargement the surface was smooth, after six or more months it tended to be irregular and showed numerous small pits. There were light peritoneal adhesions along the margins and the consistency became tougher.

No noteworthy changes were observed in the liver during the first two months except for occasional slight obscuration or exaggeration of the lobular pattern. The first definite naked-eye changes were seen in a rat killed after eleven weeks, in which the liver showed a granular surface. Thereafter the incidence of the change increased, the granularity being at times evenly distributed over the entire surface; at times limited to certain lobes or parts of lobes. The appearances recalled those of the various grades of human subacute necrosis and multilobular cirrhosis, the granular texture being of variable coarseness and the whole liver sometimes yellow, sometimes pink, or with raised nodules standing out pale pink against a dark reddish-grey background. On section, the nodular appearance was repeated, with loss of the normal lobular pattern.

Towards the end of the fourth month some of the raised nodules differed in appearance from the others in being pale greyish-white instead of pink or yellowish. It was imagined that these represented the first tumours, but histological examination did not always confirm this; it was found that these greyish-white nodules were characterised by the presence of an unusual amount of granulation or fibrous tissue, but had not necessarily proceeded to the stage of malignancy. It was not found possible to discriminate macroscopically between this lesion and the earliest stages of carcinoma, in particular the bile-duct variety; the earliest detection of carcinoma therefore rests on microscopical evidence.

When the tumours grew larger there was no longer any doubt as to their nature, and the subsequent history of the experiment is largely concerned with the increasing size and incidence of tumours. In some cases these reached very large proportions, not infrequently being larger than the rest of the organ. More often than not the tumours were multiple. Their colour might be grey, pink, yellowish or mottled, their consistency firm or soft; the amount of necrosis and hæmorrhage, as might be expected, was greatest in the largest tumours. It has not so far been possible to distinguish with confidence between liver-cell and bile-duct carcinoma by naked-eye observation, although the liver-cell tumours were more often large and of soft consistency, and in the case of the smaller firm tumours there was a suggestion that the liver-cell growths more often had a yellowish tinge.

In addition to solid tumours, many of these livers showed cysts, usually multiple and sometimes very numerous. The cysts were small, rarely more than 3 mm. in diameter, and when superficial had a bluish-grey colour. On section they contained serous fluid, sometimes faintly blood-stained, and were multilocular. These are the cystadenomata of the histological section.

In a few instances the liver displayed no surface granularity but showed increased toughness of consistency on section and

obscuration of the lobular pattern. These were the livers in which microscopy demonstrated predominantly centrolobular changes.

After eight months in the first experiment, when the administration of butter-yellow was discontinued, the macroscopical appearances of the livers, apart from the tumours, gradually reverted to normal. Only a few animals were killed during the ninth and tenth months, but in the eleventh month, when nineteen animals were examined, the majority of the livers appeared normal and the remainder only showed trivial or doubtful granularity in places. Nevertheless eight of these livers (42 per cent.) showed solid tumours, and ten (53 per cent.) contained cysts. There is thus evidence that the cirrhotic process is capable of retrogression after having reached a stage at which tumour formation occurs.

At no time did any of these rats show more than trivial amounts of ascites and varices of the portal-systemic venous anastomoses were never detected. It would seem that with butter-yellow either the cirrhosis does not cause sufficient portal obstruction to produce these effects, or the development of malignancy finishes the story before there is time for them to develop. On the whole, the first explanation seems the more likely.

Microscopical changes

First month. Of the 3 examined towards the end of the first month one liver was normal, one showed a possible increase of lymphoid tissue in the portal systems and one definite changes limited to the immediate vicinity of the portal systems, the connective tissue of which showed increased cellularity due to the accumulation of lymphocytes, large mononuclears and a few polymorphs. Extending for a short distance out from the limits of the portal tract were elongated fusiform cells of histiocyte type. The parenchyma in immediate relation to these cells showed evidence of degeneration, the liver cells being shrunken, with strongly eosinophilic cytoplasm devoid of glycogen and a tendency to nuclear pyknosis. Only a margin one or two cells thick was affected: the remainder of each lobule appeared normal, apart from occasional mitoses.

The spleens of these rats were very congested but showed no other change.

Second month. Of 8 rats examined, 6 showed changes limited to the portal tracts and their neighbourhood, in 1 the visible changes were limited to the central part of the lobules, and in 1 there were both peripheral and central changes. Among the 6 with peripheral lesions, 1 showed cellularity limited to Glisson's capsule; the remaining 5 showed periportal lesions of increasing intensity (fig. 1). Spreading out from the portal systems were

cellular strands containing some or all of the following elements:—lymphocytes, histiocytes, plasma cells, polymorphs and mononuclears of the polyblast (Maximow) type. In some cases the fusiform histiocytes occurred in double rows and at a later date this appearance led to difficulty in deciding whether the resulting structures consisted of histiocytes, vascular endothelium or even regenerated bile-ducts. Not infrequently in these periportal cellular zones there were isolated liver cells cut off from the main parenchyma. These showed the same evidence of degeneration as the narrow peripheral zone of the lobules proper, *i.e.* eosinophilic cytoplasm, absence of glycogen, fatty degeneration (the cell being packed with innumerable tiny droplets of fat) and nuclear pyknosis, as well as great variation in size and staining between individual nuclei (fig. 2). Other parts of the lobule were in general normal, showing various grades of fatty and glycogenic infiltration, though occasionally there was evidence of general damage to the hepatic parenchyma. In one rat the centres of the lobules were congested, and frozen sections showed typical fatty degeneration of the central third. In another, degeneration of the central cells was combined with cellular infiltration of the portal tracts.

Three of these rats showed early fine fibrosis of the spleen. The histological characteristics of this lesion will be described later (*vide* fourth month).

Third month. Eleven rats were examined: 1 displayed no morphological changes in the liver; in 10 the lobular changes were peripheral. The peripheral lesion had become progressively more advanced, the periportal granulation tissue extending further into the lobule and in many places becoming continuous from one portal system to another (fig. 3). Its composition was much the same as before, there being as yet no noteworthy amount of collagen, but it was now often extremely vascular. This vascularity took the form of large, sometimes almost cavernous, blood spaces lined only by a single layer of endothelium. The inclusion of sequestered liver cells in the granulation tissue was now a conspicuous phenomenon, and there can be little doubt that it was from such foci that there arose the non-architectural regeneration nodules which from now on became increasingly evident (fig. 4). In one of this group liver cell regeneration was already sufficiently advanced to justify the term "nodular hyperplasia," and in naked-eye appearance it was already granular (*cf.* fig. 5). During the third month proliferation of undoubted bile-ducts in the periportal granulation tissue was seen in a few cases. It has already been mentioned that this interpretation can be put, probably wrongly, on other appearances. The peripheral cells of the surviving part of the lobules showed the usual degenerative changes and at this time there was a tendency for more widespread damage of the rest of

LIVER CHANGES INDUCED BY BUTTER-YELLOW

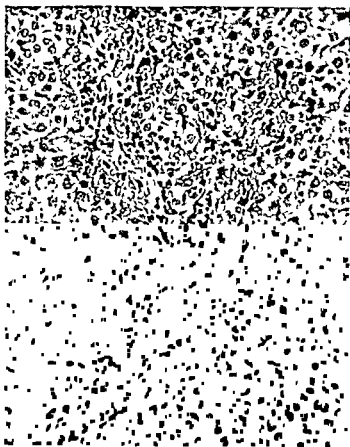


FIG. 1.—During second month of treatment. Lymphocytes and histiocytes spreading out from a portal system. The adjoining liver cells are shrunken and degenerate and show nuclear pyknosis. $\times 200$.



FIG. 2.—Second month. Degeneration of periportal parenchyma, showing (below portal vein) an abnormal, greatly enlarged, hyperchromatic liver cell nucleus. Note solid appearance of periportal liver cells, due to absence of glycogen. $\times 300$.

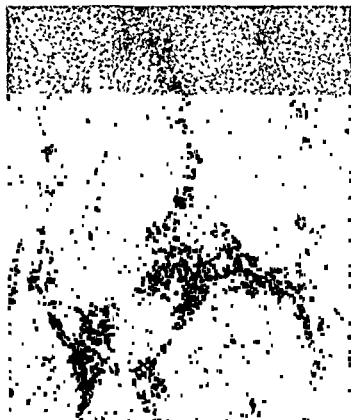


FIG. 3.—Third month. Cellular granulation tissue extending from one portal system to another. Individual liver cells and small groups of liver cells have been cut off by the proliferating granulation tissue. $\times 50$.



FIG. 4.—Third month. Non-architectural regeneration nodule surrounded by granulation tissue in upper part of field; the surrounding parenchyma appears to consist of the remains of the old lobules. The lower part of the field is still practically normal. $\times 75$.

the lobule, the cells being deformed and disarranged and their nuclei showing considerable variation in size and staining. A common type of staining change was an increase in density of the nuclear membrane and greater prominence of the chromatin nodes, giving the nucleus a speckled appearance. Pyknosis and karyolysis were also seen.

In the spleen, evidence of early fibrosis was found in 8 cases. It is of interest that these included the rat in which there was no morphological evidence of liver damage.

Fourth month. Of 15 rats examined, 9 showed peripheral, 3 central and 3 combined central and peripheral lesions. As the normal architecture was now being progressively destroyed, it was considerably more difficult than heretofore to make this classification. Of those in which the lesion appeared to originate in the portal systems, the picture represented an advance in degree of changes previously described. From now on, appreciable amounts of collagen were added to the periportal granulation tissue. Regeneration nodules of liver cells without lobular structure were now in many cases larger than the surviving remnants of the old lobules; the individual cells of these nodules were similar to those of the old parenchyma, the main mass of them showing variable grades of fatty and glycogenic infiltration, while the outside cells in contact with the granulation tissue showed fatty degeneration, absence of glycogen, and necrosis. Proliferation of bile-ducts was now a conspicuous feature, sometimes reaching an extent which might justify the description adenomatous (fig. 6). In two cases small foci had reached the stage of bile-duct carcinoma, but it will be more convenient to consider the tumours in detail later. The granulation tissue also contained a moderate amount of amorphous brown pigment, much of it in phagocytes, which was probably altered blood, though only some of it gave the Prussian blue reaction.

The histological picture as a whole at this stage suggested that the parenchyma was being as it were progressively eroded from the periphery of the lobule where it was in contact with the periportal granulation tissue, while at the same time regeneration nodules of liver tissue and masses of regenerating bile ducts were growing within and expanding the granulation tissue to form new trabeculae unrelated to the lobular structure of the liver. This condition of nodular hyperplasia only differed from the common type of cirrhosis in the relative scantiness of collagen and larger size of the regenerated bile-ducts. In 6 of these 9 rats the liver was predominantly non-architectural. In the three livers in which damage was limited to the centre of the lobules, the cells near the centre showed the usual manifestations of degeneration and necrosis. In one case this was associated with hæmorrhage, giving the "nutmeg-liver" picture; in another there was some infiltration with lymphocytes

and plasma cells around the intralobular veins. In two of the livers with both peripheral and central involvement, minor amounts of periportal granulation tissue were combined with central degeneration and (in one case) hæmorrhage; in the third, well marked nodular hyperplasia was associated with hyaline degeneration of the walls of the intralobular veins.

Eight of the 15 fourth-month rats showed fibrosis of the spleen, 2 in an advanced stage. The amount of fibrosis did not necessarily correspond with the magnitude of the liver changes and it was associated with both central and peripheral hepatic damage. The fibrosis might involve either the pulp or the Malpighian bodies or both (*cf.* figs. 7 and 8). It appeared to start in the adventitia of the veins, from which it spread to involve the pulp; or in the region of the peripheral ring of "reticulo-endothelial" cells found in the Malpighian body of the rat, from which it might spread inwards to involve the follicle itself or outwards to involve the pulp. It was nearly always most marked in one or both of these two sites. When the pulp became involved the collagen was found in delicate strands in the intersinusoidal tissue. This change, with the accompanying congestion, had the effect of accentuating the sinusoidal pattern of the pulp. The appearances were very similar to those seen in human cases of hepatic cirrhosis, including Banti's disease. When the Malpighian bodies were affected, there was replacement of lymphoid tissue by relatively dense collagen, so that the process was more apparent with low magnifications than in the case of the pulp. In both sites the collagen was laid down without any sign of fibroblastic activity. In the most advanced stages there appeared to be a multiplication of trabeculæ; this may have been more apparent than real and have resulted from approximation of trabeculæ as the spleen shrank in later stages, when it might also contain quite considerable fibrous scars (fig. 9). Myeloid metaplasia was seen in a number of the spleens, but should not be stressed as it is a common finding in rodents' spleens in a number of conditions.

Fifth month. Seventeen rats were examined; in 12 the lesion was primarily peripheral, in 2 central, in 2 both peripheral and central, and in 1 there was general degeneration of the parenchyma without proliferation of the interstitial tissues. In the 12 with peripheral lesions, there was nothing new in the nature of the changes, but in general they were more extensive. The amount of collagen in the trabeculæ was increased, though in most cases these were still very cellular and vascular. A well marked non-architectural nodular hyperplasia was present in 7 cases, in the other 5 the granulation tissue still followed to a large extent the lobular pattern. The cells in contact with the granulation tissue, both old parenchyma and regeneration nodules, still showed typical

CHANGES IN LIVER AND SPLEEN INDUCED BY BUTTER-YELLOW

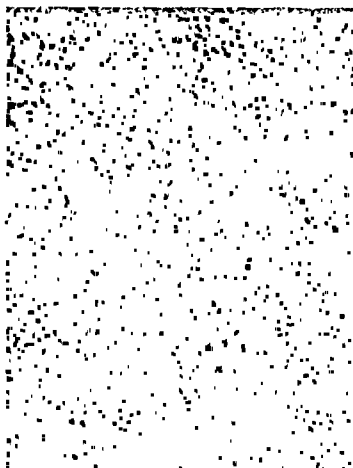


FIG. 5.—Fourth month. Nodular hyperplasia. The lobular pattern is largely replaced by non-architectural regeneration nodules, separated by trabeculae of granulation and fibrous tissue. $\times 65$.

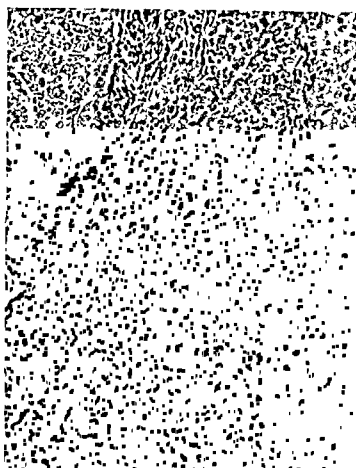


FIG. 6.—Fourth month. "Adenomatous" hyperplasia of bile-ducts. $\times 160$.

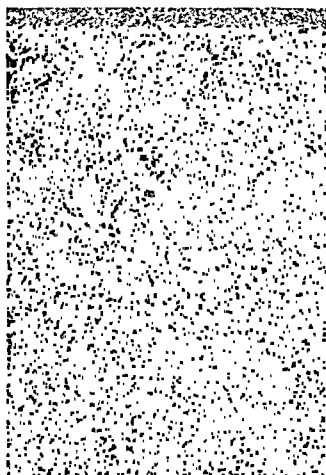


FIG. 7.—Fifth month. Fibrosis of spleen, mainly affecting the pulp, but encroaching upon, and reducing the size of, the Malpighian bodies. $\times 65$.



FIG. 8.—Sixth month. Fibrosis of spleen, affecting the "reticulo-endothelial" region of the Malpighian bodies. $\times 110$.

degenerative changes, though sometimes this was less conspicuous in the regeneration nodules. Bile-duct proliferation was well marked, frequently reaching adenomatous proportions, and in 2 cases there was focal carcinoma. Cystadenomata were seen in 3 cases. Mitotic figures were frequent in the liver cells. Of the two livers with central lesions, one was a surprisingly acute-looking lesion for this date in the experiment, the only change being fatty degeneration with absence of glycogen in the centrolobular liver cells. In the other, degeneration was more widespread and there were hæmorrhages in the centre of most lobules. In two combined lesions a well marked portal lesion reaching the stage of non-architectural nodular hyperplasia was associated with acellular fibrosis round the central veins.

Splenic fibrosis was present in the majority of the fifth-month cases and was well marked in over half. Again the degree of development of the splenic lesion did not follow that of the hepatic; in the case without connective tissue proliferation in the liver, the spleen showed advanced fibrosis.

Sixth month. Twelve rats were examined; in 8 the lesion appeared to be primarily peripheral, though it was now becoming more difficult to analyse the sometimes complicated histological picture of increasing nodular hyperplasia. The changes were similar to those already described, but more advanced. Tumours were more numerous and included the first examples of liver-celled cancer. Three rats showed lesions originating in the region of both portal and intralobular veins. In one of these there was well marked outgrowth of granulation tissue from the central vein; the others showed hæmorrhage or acellular fibrosis in this area. In one liver there was but little evidence of proliferation of the connective tissue elements, but the parenchyma had the appearance of advanced non-architectural regeneration.

All save three of these cases showed splenic fibrosis, one of the exceptions being the last-mentioned.

Seventh month. Twenty-seven rats were examined; in a considerable number the advanced nodular hyperplasia made it difficult to observe directly from what part of the lobules the process had started. With this reservation, it appeared that the lesion was of peripheral origin in 25, central and combined in one each. In both the latter the central changes were degenerative. It seems probable, from a consideration of the earlier results, that some of the other livers must at some time have had changes related to the interlobular veins.

Splenic fibrosis was obvious in the majority, sometimes reaching the stage of considerable collagenous scars (fig. 9), and it was now particularly noteworthy that the extent of splenic fibrosis was not directly related to the amount of liver change.

Eighth month. It was during this month that the administration of butter-yellow was discontinued (after 232 days) in order to investigate the tendency to retrogression of the changes. Nineteen animals in all were killed (five during butter-yellow treatment), and the changes found do not necessitate description additional to that for the previous month.

Ninth month. Two rats were examined; both showed liver tumours. As regards the rest of the organ, the primary lesion was peripheral in both and one showed nodular hyperplasia.

Tenth month. Three rats were examined; 2 showed liver tumours. In one of these, many parts of the rest of the liver were histologically normal; in the other there was evidence of parenchymatous regeneration, both with and without associated cirrhosis, and areas of mid-zonal necrosis in the lobules. In both, however, there was evidence of persistent cirrhosis in relation to the tumour deposits. In the third rat there was one small focus of persistent cirrhosis, but in most places the lobular pattern was intact and the only abnormal feature was a slight acellular fibrous increase round both venous systems; proliferation of bile-ducts was nowhere seen. Splenic fibrosis was present in all three.

Eleventh month. Nineteen rats were examined; in 8 the liver contained solid tumours and in 10 there were cysts, but in no case were the macroscopical or microscopical appearances of the rest of the liver tissue similar to those of three months previously. In the majority of cases the liver pattern had returned practically to normal, though evidence could be found pointing to changes at an earlier date. This most frequently took the form of a slight acellular fibrous increase round the veins. A further indication was that in some cases the normal radial arrangement of the cells in the lobule was not fully re-established, and in particular occasional areas were seen where the liver cells were obviously regenerating, being smaller than the other parenchymatous cells, patternless in disposition and showing a number of mitoses. In two livers there was lymphoid hyperplasia in the portal systems, but this is not an uncommon finding in the normal rat. In a few cases there were traces in patches of the old cirrhosis, while the rest of the organ was normal. Only in one case was any periportal cellular granulation tissue seen, and that in minimal amounts.

But perhaps the most important indication of previous cirrhosis was its persistence in relation to the tumours which were found in otherwise normal livers. It might have been argued in certain cases that such appearances were due to stroma-formation in the tumours themselves, but it is not believed that this is the explanation. In the first place, the composition and trabecular distribution of the fibrous and granulation tissue strongly recalled the cirrhotic process, and in the second, one could detect islands of

CHANGES IN LIVER AND SPLEEN INDUCED BY BUTTER-YELLOW



FIG. 9—Seventh month. Considerable mass of fibrous scar tissue in the spleen. $\times 65$



FIG. 10—Bile duct carcinoma $\times 75$.



FIG. 11—Bile-duct cystadenoma. The alveoli are lined by cuboidal epithelium. $\times 100$.



FIG. 12—Bile-duct cystadenoma. The alveoli are lined by flattened cells, and the appearance recalls that of the human cavernous hemangioma, except for the absence of blood. $\times 60$.

cirrhotic liver tissue which had become incorporated in the carcinomatous mass. It would therefore appear that at the time cancer arises the cirrhosis has not yet reached an irreversible stage, and that retrogression of the cirrhotic process is prevented, or at any rate retarded, at the site of a tumour.

Splenic fibrosis was present in 13 of these rats. Whether regression would ultimately have occurred cannot be stated, but if so it must be a considerably slower process than in the liver.

The tumours

The naked eye appearance of the tumours has already been described. In addition to the solid tumours, the cysts can conveniently be considered here, they are regarded as originating in the bile ducts. There are thus three broad categories of tumours—bile-duct carcinoma (cholangioma), bile duct cystadenoma and liver cell carcinoma (hepatoma). All possible combinations in any one liver were found (table II).

TABLE II
Incidence of the three types of tumour

Group	Type of tumour	No of tumour bearing animals
A	Bile duct carcinoma only	13
B	Bile duct cystadenoma only	13
C	Liver cell carcinoma only	5
D	A+B	9
E	A+C	4
F	B+C	5
G	A+B+C	7
	Total tumour bearing animals	56
	Malignant tumour bearing animals	43

Bile duct carcinoma These arose in the areas of bile duct proliferation in the trabeculae of granulation tissue. It would be possible to describe a prior stage of bile duct adenoma, but this would be somewhat artificial, as it is not possible to draw any sharp distinction, other than quantitative, between regenerative and neoplastic proliferation until the stage of malignancy or cystic growth is reached. The earliest bile duct carcinoma was detected after 111 days treatment.

The carcinomatous foci were easily distinguishable from the non malignant tissue (fig 10). The cells were irregular in shape and size and the cytoplasm was frequently strongly haematoxyphile. The nuclei were hyperchromatic and indiscriminately placed in the cell, mitoses were frequent. The contours of the tubules might be irregular and layering of the cells sometimes occurred, sometimes the acini were solid, when confusion with liver cell growth was

possible. In some cases the cells had disappeared and been replaced by faintly hæmatoxyphile material, so that the picture was that of mucoid carcinoma. One had an impression that this mucoid change occurred most often when the stroma of the growth was more than usually dense. The appearance seems to be what Kinoshita (1937) regards as a "myxomatous" change due to suppression of epithelial growth by fibrosis.

The incidence of bile-duct carcinoma did not appear to be related to the amount of simple hyperplasia of bile-duct epithelium present; sometimes almost all the proliferating bile-ducts were already malignant. The tumours were more often multiple than not. As can be seen from the table, they were associated with other varieties of tumour in 60 per cent. of cases.

Bile-duct cystadenoma. These interesting growths were sometimes obviously derived from bile-duct epithelium and formed relatively large cavernous spaces lined by cuboidal epithelium (fig. 11). In other cases the lining epithelium was flattened and resembled endothelium, but as all intermediate shapes of cell have been seen, there was good reason to believe that they were all of similar origin. The stroma consisted of well formed slender collagen fibres and not infrequently contained fragments of hepatic parenchyma, sometimes in an apparently viable condition (fig. 12). The earliest cystadenoma was found after four months. An interesting aspect of many of these cystadenomas was their resemblance to the common cavernous hæmangioma of the human liver. This was particularly noticeable when the lining cells were of the flattened "endothelial" type. Only in two instances, however, was there any blood in the alveoli.

Liver-cell carcinoma. The tumours included under this head were all frank tumours of indisputable malignancy. It was sometimes an open question whether a given lesion should be regarded as an extreme example of active non-architectural regeneration or as neoplastic; in such cases the former interpretation was adopted for statistical purposes.

Liver-cell cancer appeared to have originated as a further stage of the regenerative process. As in the case of bile-duct tumours, it was frequently multifocal in origin. As might be expected, it has not been possible to decide whether any cancerous focus had started from a single cell or from a group of cells. In the earliest nodules, groups of non-neoplastic liver cells were incorporated in the young tumour mass, a finding which would tend to support the second view unless the tumour cells possess great infiltrative properties from their earliest stages. In suitably stained sections the cells of these tumours did not contain glycogen, but here and there a glycogen-containing cell could be seen; these were probably sequestered parenchymatous cells.

LIVER CHANGES INDUCED BY BUTTER-YELLOW

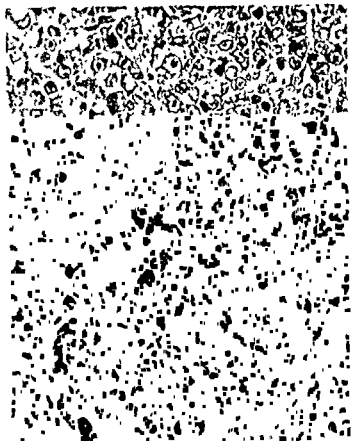


FIG. 13.—Liver-cell carcinoma; normal liver in left upper quadrant. Anaplasia and mitoses in the tumour. $\times 250$.

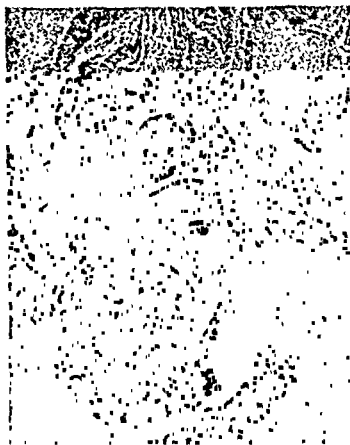


FIG. 14.—Liver-cell carcinoma of alveolar structure, with considerable necrosis. $\times 50$.



FIG. 15.—Liver-cell carcinoma. Structure not unlike normal liver. Numerous large blood sinuses. $\times 90$.



FIG. 16.—Area from liver-cell carcinoma, showing tubular structure. The cells, however, were more like liver cells than bile-duct epithelium. $\times 50$.

The morphology of the liver-cell carcinomata was very variable in different tumours and in different parts of the same tumour. Sometimes they closely resembled regeneration nodules, differing only in the absence of glycogen and in the frequency and atypicality of mitoses. At other times the cells were larger and more anaplastic, but the structure was still that of a solid polyhedral-celled carcinoma (fig. 13); such types of growth showed a greater tendency to necrosis than any of the others (fig. 14); some of them contained large blood sinuses (fig. 15). In some of the liver-cell carcinomata there were considerable amounts of tubular growth and at times this gave rise to doubt as to whether the tumour was not of bile-duct origin (figs. 16 and 17). The most helpful criterion in the most difficult sections was probably the appearance of the tumour nuclei. Liver-cell nuclei are in general relatively larger than those of bile-duct epithelium and show a somewhat firmer nuclear membrane and a more clearly marked reticulation and nodding of the chromatin; liver-cell nuclei generally have one prominent nucleolus, often acidophile, whereas bile-duct nucleoli are smaller, hardly ever acidophile and often two or three are equally prominent.

In two cases liver-cell cancers had invaded the portal veins (fig. 18). Metastases were present in these cases in the cœliac and mediastinal lymph glands (fig. 19) and in the lungs in one case (fig. 20).

Liver-cell carcinoma did not appear so early as bile-duct carcinoma, the first example being found after 175 days in a liver which also contained bile-duct cancer. They were in general more actively growing tumours and attained a greater size.

Discussion

It would appear from the present work that the primary effect of butter-yellow on the liver is of a damaging nature and that the proliferation of liver and bile-duct cells which follows is regenerative in nature. Such a view is supported by short term experiments in collaboration with Dr D. E. Price on the effects of large doses of butter-yellow. The earliest evidence of proliferation is usually found, not in the parenchymatous cells, but in the connective tissue of the portal tracts, from which granulation tissue extends into the substance of the lobule. At this time the adjacent liver cells are definitely degenerate. The subsequent course of events appears to be similar in many respects to what happens with a variety of agents which produce degeneration and necrosis in short term experiments and cirrhosis after prolonged administration. An impressive list of such substances is found in the review of the subject of experimental cirrhosis by Moon (1934). At the same time it is clear from such investigations that the establishment of cirrhosis does not in itself lead to cancer.

It was shown by Cameron and Karunaratne (1936) that when the histological picture of cirrhosis was reached with carbon tetrachloride, there was still a period when the process was completely reversible, with return to a normal liver, though later an irreversible cirrhosis was reached. In view of these observations, butter-yellow administration was stopped when well developed nodular hyperplasia was reached in the present experiments, and it has been shown that both types of liver cancer can arise when the cirrhotic changes are still in the reversible phase. Indeed, one is not convinced that an irreversible cirrhosis can be established with butter-yellow; it may well be that the intrusion of cancer would terminate the experiment too soon in every case. In the meantime it seems desirable that a comparative histological examination should be made of the cirrhosis produced by carcinogenic azo dyes and other agents.

It seems possible that part of the explanation of the fact that butter-yellow is carcinogenic while other cirrhosis-producing agents are not may rest on the fact that regenerative hyperplasia of liver and bile-duct cells occurs quite early in the course of butter-yellow administration, and that the associated fibrosis does not attain as great a density as with the other agents, as far as can be judged from illustrations and the few sections which have been seen by the author. It has previously been suggested (Orr, 1938, 1939) that alterations in the direction of rarefaction of the associated connective tissue are of importance in chemical carcinogenesis in the skin and subcutaneous tissues. Moreover, according to Yoshida (1935), *o*-aminoazotoluene produces cirrhosis but not cancer in rabbits, while hepatoma can occur without cirrhosis in the rat. It should be mentioned that the writer has had the opportunity of examining sections from several rats treated with the latter dye by colleagues, and has always seen cirrhosis in association with developing tumours, though the liver might not be hob-nailed.

The changes in the spleen are of considerable interest, having regard to the differences of opinion which exist as to the relationship of the hepatic and splenic changes in the so-called Banti's disease. It was clear in the present investigation that the primary congestive enlargement of the spleen was not the result of cirrhosis, which developed much later. This does not necessarily mean that it was not due to intrahepatic obstruction to the portal circulation, but the usual other indications of portal obstruction were absent. Later, when fibrosis of the spleen developed, its extent bore no relationship to the extent of change in the liver, severe grades of splenic fibrosis accompanying relatively normal livers and *vice versa*. There are no reasonable grounds for not believing that the changes in both organs were brought about by the same agent, butter-yellow, but it seems doubtful to what extent they were inter-related.

LIVER CHANGES INDUCED BY BUTTER-YELLOW



FIG. 17.—Carcinoma of tubular structure, but the lumen of the large tubule contains a mass of what were believed to be degenerate liver cells. $\times 200$



FIG. 18.—Liver-cell carcinoma (above) permeating the lumen of a portal vein. Normal liver below. $\times 90$



FIG. 19.—Metastatic liver cell carcinoma in coeliac lymph gland. $\times 90$.



FIG. 20.—Metastatic liver-cell carcinoma in lung. $\times 65$.

At one stage of the present experiments a high proportion of the rats showed chronic ulceration of the caecum, with replacement of all its layers by fibrous and granulation tissue. Analysis of the results, however, does not suggest that this lesion had any aetiological relationship to the hepatic or splenic changes. In any case it is not necessary for carcinogenesis that the butter-yellow should reach the liver via the alimentary canal, Nakahara and Fujiwara (1938) having obtained positive results by intraperitoneal injection.

It is not clear why the changes in the present series developed so much more slowly than those obtained by Kinoshita (1937). It has already been mentioned that the diet was changed from wheat, maize and oats to rice for the later group of animals, since the work of Fischer-Wasels (1937) and Ando (1938) suggested that this would be more effective. The acceleration of liver changes so obtained was negligible, though the fact that *unpolished* rice was used may have contributed to this. At the time it was believed that this was the normal food used by the Japanese workers, but in a recent paper, Nakahara, Mori and Fujiwara (1939) describe experiments with butter-yellow, using as food polished rice with the following supplements:—dried liver, purified protein, butter, inorganic salts, vitamins B 1, B 2 and B 6, nicotinic acid, and all of them (except liver) together. In all cases, with the exception of those receiving liver, the changes advanced more rapidly than in the present experiments. The addition of liver to the diet, on the other hand, appears to have had a remarkable inhibiting power on the carcinogenic process.

Summary

Butter-yellow (*p*-dimethylaminoazobenzene) was added to the food of 200 white rats and the course of the histological changes in the liver followed in 136 which were killed at intervals throughout the experiments.

The usual sequence was proliferation of connective tissue cells in the portal systems, extension of granulation tissue from the latter into the parenchyma, with degeneration of the contiguous liver cells, atypical regenerative proliferation of bile-duct and liver epithelium leading ultimately to non-architectural nodular hyperplasia and a macroscopically hob-nailed liver, in which in a certain proportion of cases tumours arose.

In a few animals the predominant changes were at the centres of the lobules, but in these tumour formation did not occur.

Three types of tumour were found: liver-cell carcinoma in 21 animals, bile-duct carcinoma in 33 and bile-duct cystadenoma in 31. All combinations occurred, and the total number of tumour-containing livers was 56, malignant tumours being present in 43.

Butter-yellow administration was stopped after 232 days and the livers then returned to normal, apart from the presence of tumours, in 2-3 months. It has been shown that cancer can arise while the cirrhosis is still in the reversible phase, and it seems unlikely that an irreversible cirrhosis could ever be obtained with this agent in the rat.

The rate of development of the hepatic lesions was slower than has been reported by Japanese workers.

The spleen also showed important changes—congestion followed by fibrosis of the pulp or Malpighian bodies or both. The early splenic congestion preceded major hepatic changes. The degree of splenic fibrosis was not always commensurate with the amount of hepatic change.

It is believed that the primary effect of butter-yellow on the liver parenchyma is destructive and that the proliferative changes are of the nature of regeneration.

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THE OCCURRENCE IN NORMAL MICE OF PLEURO PNEUMONIA LIKE ORGANISMS CAPABLE OF PRODUCING PNEUMONIA

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(PLATE XLVIII)

THERE have been several recent reports of the discovery in mice of filter passing organisms capable of growing in artificial media and resembling morphologically the organism of bovine pleuro pneumonia. They were accidentally discovered during routine passage of viruses, or of material suspected of containing viruses, by intracerebral and intra ocular inoculation (Findlay *et al*, 1938, Sabin, 1938 *a* and *b*, 1939*b*). Such contamination of a virus during passage by pleuropneumonia like organisms could only be explained by the presence of the latter in a latent form in certain normal mice. This was shown to be true by Sabin (1938*b*), who obtained an organism of this type by direct culture from a normal mouse's brain. Subsequently he isolated other strains from the conjunctival sacs, nose and lungs (1939*b*). Similar organisms were recovered by Sullivan and Dienes (1939) from pneumonic lesions resulting from intranasal inoculation of mice with suspensions of normal mouse lungs.

The presence of pleuropneumonia like organisms in the nose, whence they can be carried to the lungs with the production of pneumonic lesions, is a fact of practical importance, for intranasal inoculation of mice is being increasingly used for the investigation of viruses. It is the purpose of this paper to describe organisms of this type capable of producing pulmonary and other lesions and occurring in two stocks of mice extensively used for virus research. The properties of the strains isolated are fully reported as they appear to differ in many respects from the organisms previously described.

Isolation of the original strains

The investigation that led to the discovery of the organisms here described was initiated by Dr C. H. Andrewes, who inoculated two batches of mice intranasally with diluted vesicular fluid from

two cases of chicken-pox. One mouse in the first batch had consolidated lesions in the lungs. With the original mice of the second batch no lesions appeared, but one out of six mice inoculated with their lungs developed a similar lesion. Suspensions of the affected lungs were capable in each case of reproducing the disease in a high proportion of normal mice inoculated intranasally and the agent responsible was transmissible by passage in this way. It therefore became necessary to investigate its nature.

Material filtered through gradocol membranes with average pore diameters between 0.71 and 0.85μ still produced pneumonia, although in a smaller proportion of animals. After passing filtered material several times it was found that the unfiltered lung suspensions were sterile when cultured on ordinary media. It was therefore decided to use cultural methods suitable for the isolation of pleuropneumonia-like organisms. The affected lungs were finely minced with scissors, ground up in a mortar with powdered pyrex glass and a 10 per cent. suspension made in a mixture of equal parts of broth and saline. This was centrifuged for 20-30 minutes in the laboratory centrifuge and 0.5 and 0.1 c.c. inoculated each into 5 c.c. of broth containing 10 per cent. horse serum. The cultures were incubated for at least 14 days at 37° C. before being discarded. If an opalescence developed subcultures were made. By these means organisms morphologically resembling the organism of bovine pleuropneumonia were isolated from lung suspensions on the occasion of five consecutive passages of each of the two transmissible strains. Proof that these organisms were responsible for the pneumonic lesions was obtained when it was found that cultures alone were capable of producing the lesions.

The lung lesions produced by passage appeared to develop slowly. They were more frequently found in mice killed 14 days after inoculation than in those killed after 7 days. On the fourth day lesions were even fewer and passage from these lungs was unsuccessful. Therefore the tenth day was chosen for killing the mice for passage or for estimating the frequency of lesions. Even at 14 days, death from pneumonia was rare and the mice usually appeared healthy, although they might have a large portion of their lungs consolidated. In the earlier passages the percentage of takes was low and rather irregular; later, after a considerable number of passages, 5 or 6 out of a batch of 6 mice would have lesions.

Infective lungs dried *in vacuo* and left for at least two months retained their infectivity. Storage in 50 per cent. glycerol in saline for one week, however, destroyed it.

Isolation of the organisms from normal mice

It appeared probable that the strains of pleuropneumonia-like organisms so far isolated had come from the mice used for intranasal inoculations. Attempts were therefore made to isolate them from normal mice of the same stock. This stock, which was used throughout the investigation except on the occasions, mentioned

later, when Swiss mice were used, is a mixed one bred at the Farm Laboratories of the Medical Research Council. The animals chosen were young mice weighing 14-16 g.

At first cultures were made directly from the conjunctivæ, nose and lungs on serum agar plates and in serum broth. No pleuropneumonia-like organisms were isolated. Although they had been successfully employed by Sabin (1939b), these direct cultural methods were abandoned after a few attempts for the following reasons. The strains which had already been isolated failed to form surface growths of appreciable size on serum agar plates and thus plating was unlikely to prove effective for isolation of the organisms. Cultures made directly in serum broth from the nose and eyes gave, on the other hand, profuse bacterial growth. It was therefore decided to inoculate normal mice intranasally under ether anæsthesia in the hope of encountering organisms which would produce pulmonary lesions, as had happened in the original experiments.

Three normal mice were killed and suspensions of their lungs made in broth-saline in the manner already described. After centrifugation the supernatant fluid from each suspension was inoculated into 12 mice. When killed ten days later 1 mouse in one batch and 2 in another showed typical areas of consolidation in the lungs. There were therefore 3 out of 36 mice with pulmonary lesions identical in appearance with those produced by the pleuropneumonia-like organisms, as well as several with abscesses due to bacterial infection and others with small grey patches in the lungs, not definitely pneumonic. Attempts were made to pass the infection from the affected lungs to other mice. A suspension of one lung produced pneumonia in 6 out of 6 mice and in a second passage from these 4 out of 5 were positive. Passage from the other two lungs was also successful, but the proportion with lesions was less, 2 out of 6 and 1 out of 4. Cultures in serum broth were made from suspensions of the originally affected lungs and from the consolidated lungs resulting from the passage experiments. Three strains of pleuropneumonia-like organisms were isolated and thus the presence of these organisms was demonstrated in normal mice. It is worthy of mention that the culture from the passage material which gave 6 out of 6 and 4 out of 5 positives, produced lesions in 4 out of 6 mice when inoculated intranasally.

Description of the strains of pleuropneumonia-like organisms isolated

Five strains have been isolated and examined. Strains 1 and 2 were recovered from the lungs of mice used for passage from the lesions encountered in the original experiments with chicken-pox material, strains 3, 4 and 5 from normal mice as described.

When examined microscopically by dark-ground illumination,

cultures of all five strains had the peculiar pleomorphic appearance characteristic of the organism of bovine pleuropneumonia. In young cultures there were to be seen small granules, ring forms of various sizes, forms showing filamentous budding, and thin bacillary bodies with several rounded swellings along their length. In older cultures there was a larger proportion of the filamentous elements.

Satisfactory growth was obtained in Hartley's tryptic digest broth of pH 7.8-8.0, to which had been added 10 per cent. of horse serum. As a routine, subcultures were made with inocula of 0.1 c.c. Growth was usually first apparent on the second or third day. Only rarely did it appear on the first day, and if the number of viable organisms in the inoculum was small, it might be delayed for as long as 14 days. It was evident as an even opalescence, which slowly became denser, with the formation of a little fine granular deposit at the bottom of the tube. This could be disturbed by shaking, when it tended to rise spirally upwards, thus serving as a useful indication of the presence of growth.

It was found that subcultures were most successful when made on the third to the fifth day of incubation, soon after growth had appeared. Even so it has been found difficult to maintain the strains.

Although growth in fluid media was fairly satisfactory and usually quite profuse, that on solid media was extremely scanty. Even after more than a week's incubation on agar plates containing 10 per cent. of horse serum, there was only a slight roughening of the surface due to confluent growth. Separate colonies of sufficient size to be picked off for subculture were not obtained. The whole of the investigation has therefore been carried out with cultures in fluid media.

The organisms grew equally well under aerobic and anaerobic conditions. No improvement was produced by incubation in an atmosphere of 5 per cent. carbon dioxide. Growth occurred at 30° C., but it was slower and less profuse than at 37° C. and none occurred at 22° C. The broth originally used for making the culture media had a pH of 7.6-7.8; it was found that growth was improved by using a more alkaline broth. That used later had a pH of 7.8-8.0, although even more alkaline media (up to pH 8.6) were equally effective. The substitution of ox for horse serum was of no benefit. There was slight growth in plain broth containing Fildes' peptic digest of blood; after the addition of 20 per cent. horse serum it was equal to that in ordinary serum broth and similar to that obtained in glucose broth with added serum and catalase. There was only scanty growth in peptone water containing 20 per cent. serum. None occurred in digest broth without added serum. The addition of yeast extract or cystine caused no improvement nor

did the sodium salts of lactic, formic, citric, succinic and fumaric acids.

All the strains were tested for their ability to ferment glucose, fructose, galactose, xylose, arabinose, rhamnose, maltose, sucrose, lactose, raffinose, dextrin, inulin, salicin, mannitol, dulcitol and glycerol, but production of acid from any of these could not be definitely established.

Resistance. A temperature of 55° C. for 15 minutes killed the organisms. They resisted temperatures of 45° and 50° C. for 15 minutes but not for 30 minutes. They withstood drying *in vacuo* for at least two months.

Ultra-filtration analysis. Experiments were carried out similar to those of Laidlaw and Elford (1936), who filtered young cultures of the pleuropneumonia-like organisms isolated from sewage through a series of gradocol membranes (Elford, 1931, 1938) in order to estimate their size. Cultures of strain 4, which had been incubated for 48 hours, were filtered through membranes of average pore diameter (A.P.D.) $0.8\ \mu$, after a sample had been titrated by subculturing tenfold dilutions. A sample of this filtrate was retained for a similar titration and the rest divided into 10 c.c. portions and filtered through membranes of smaller A.P.D. Each filtrate was titrated. In a typical experiment the concentration of organisms in the culture was found to be 10^8 per c.c., which was reduced to 10^5 per c.c. by filtration through the membrane of A.P.D. $0.8\ \mu$. After filtration through membranes of A.P.D. 0.53 and $0.45\ \mu$ the concentrations were 10^3 and 10^2 per c.c. respectively. No growth was obtained with filtrates from membranes of A.P.D. 0.33 and $0.25\ \mu$, even though 5 c.c. of each were incubated. The filtration end-point is thus about $0.33\ \mu$ and the estimated size of the smallest viable forms is between 165 and 247 m μ .

These results, which demonstrate a marked variation in size of the infective bodies, with a small proportion capable of passing membranes of very fine pore diameter, are typical of the pleuropneumonia-like organisms. The smallest viable forms of bovine pleuropneumonia have been estimated to measure between 125 and 175 m μ and those of agalactia 150-200 m μ (Elford, 1938). The sewage organisms described by Laidlaw and Elford were of the same size as the organism of bovine pleuropneumonia (125-175 m μ). An organism (strain A) isolated from mice by Sabin (1938*b*) gave figures of 250-292 m μ , and another of this type which caused arthritis in rats (Findlay *et al.*, 1939) had a filtration end-point of 300 m μ (estimated particle size 150-200 m μ).

Pathogenicity for animals. Intranasal inoculation of mice was carried out with several strains. A young culture of strain 1 (second subculture) produced lesions in 3 out of 6 mice; the fifteenth subculture gave lesions in only 1 out of 5. The third subculture of

strain 2, concentrated 20 times by centrifugation, was positive in 5 out of 6, but the fifteenth subculture gave no lesions in 6 mice. There is thus a marked loss in pathogenicity after prolonged culture in artificial media. Second subcultures of strains 3 and 4 infected 2 out of 4 and 4 out of 6 mice respectively, and the seventh subculture of the latter strain 2 out of 6.

The typical lesions produced in the lungs were areas of consolidation, single or multiple and affecting the whole or part of a lobe. Frequently they were near the hilum. When the mice were killed about a week after inoculation the lesions had a greyish red colour. The surface was coarsely mottled with pale areas separated by bands of darker colour. The lesions thus differed from those of influenza, which are typically purple in colour and have a more uniform surface. When the mice were killed later, the lesions were paler and greyer, but the mottling persisted.

Many of the affected lungs were examined histologically. The lesions are of lobular distribution and consist of an exudate into the alveoli of polymorphonuclear leucocytes and macrophages. If the changes are traced from unaffected areas of the lung towards established lesions, an increase in polymorphs and macrophages in the alveolar walls is first noticed. There is then an outpouring of oedema fluid into the alveoli, followed by polymorphs, macrophages and alveolar phagocytes. These cells frequently show evidence of degeneration. The bronchi and bronchioles contain plugs of degenerate pus cells and macrophages in their lumina (fig. 1). The bronchial and bronchiolar epithelium does not appear to be affected, contrasting with the findings in influenza (Nelson, 1939; Straub, 1940).

In mice killed at the end of a week another change is evident, which later becomes a striking feature, namely marked proliferation of reticulum cells around the bronchi and blood vessels. Later these form large cellular masses, apparently by proliferation of the undifferentiated mesenchyme in the walls of the blood vessels. The cells comprising them are chiefly reticulum cells and macrophages, and there are also a few more undifferentiated mesenchymal cells, plasma cells and occasionally eosinophils; polymorphs are rare (fig. 2). Many of the cells show mitotic figures.

Mice have also been inoculated subcutaneously, intraperitoneally and intravenously with cultures of strains 1, 2 and 4. All the animals remained healthy and appeared unaffected, although observed for as long as a month. Post-mortem examination, however, revealed certain lesions. In about half the animals there were one or a few small areas of consolidation in the lungs visible to the naked eye. Microscopically, essentially the same pathological changes are found as after direct intranasal inoculation, but the lesions are much smaller and they are scattered. The proliferated

PLEUROPNEUMONIA-LIKE ORGANISMS IN NORMAL MICE



FIG. 1.—Lung of mouse inoculated intranasally. Consolidated lung and massive reticulum cell hyperplasia round a pus-filled bronchiole and a bronchial artery.
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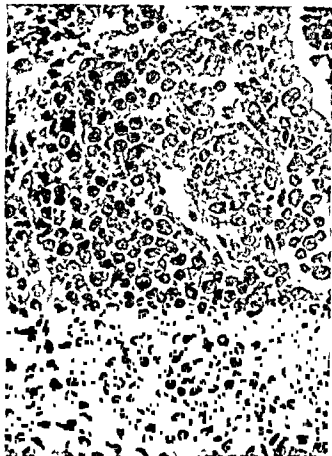


FIG. 2.—Higher power view of another part of same section, showing hyperplastic reticulum cells. Consolidated lung may be seen on the left, and there is a small portion of a pus filled bronchiole in the top right hand corner. $\times 460$



FIG. 3.—Liver of mouse inoculated intraperitoneally, showing large collections of reticulum cells. $\times 120$.



FIG. 4.—Liver of mouse inoculated intraperitoneally. Collections of reticulum cells in sinusoids and near a vein. $\times 3$

masses of reticulum cells are prominent and there is exudation of inflammatory cells into the alveoli, but amongst these, polymorphs are less frequent. The only other naked-eye finding was occasional enlargement of the spleen. Histological examination showed large follicles, but similar appearances were seen in the spleens of normal mice examined as controls. There were, however, striking changes in all the livers examined. These consist of granulation and occasional vacuolation of the hepatic cells, together with numerous collections of mononuclear cells. The latter chiefly occur beside the hepatic veins but occasionally also near the portal veins, and some are of large size (fig. 3). In a few livers there are also numerous smaller collections in the sinuses (fig. 4). Most of these cells appear to be reticulum cells, similar to those found in the lungs, and no polymorphonuclears are present. Similar appearances are not found in normal mice.

Five attempts were made to recover the pleuropneumonia-like organisms by culturing suspensions of lungs, liver and spleen, but positive results were obtained with only two lung suspensions and one splenic.

Subcutaneous inoculation of cultures alone did not produce local suppuration, but a mixture of culture and suspension of sterile agar caused abscesses at the site of inoculation; these later discharged through the skin and healed. A suspension in saline of the thick caseous pus from one of these abscesses produced fresh abscesses when inoculated subcutaneously. A second passage in this way was unsuccessful. Pleuropneumonia-like organisms were recovered from the pus in pure culture. The mice with abscesses did not show any lung lesions and the one liver examined histologically showed no abnormality. There thus appears to have been fixation of the organisms at the site of inoculation.

Intracerebral inoculation was without effect, even after mixing the culture with a suspension of sterile agar, a proceeding which was found by Findlay *et al.* (1938) to promote the virulence of the L 5 organism, the cause of "rolling disease" in mice.

Experiments have also been carried out to estimate the pathogenicity for young rats of 40-50 g. weight. Intranasal inoculation with doses of 0.1 c.c. produced a few lesions similar to those found in mice. There is, however, a form of bronchopneumonia occurring spontaneously in rats due to a pleuropneumonia-like organism (Klieneberger and Stebbins, 1937), so the significance of these lesions is difficult to estimate. One of 6 rats injected intravenously showed lung lesions of a similar type; no changes were found in the liver. Subcutaneous inoculation of plain culture was without effect, but a local abscess was produced when culture was mixed with an agar suspension.

No lesions were produced in four guinea-pigs of about 150 g.

weight inoculated intranasally with 0.25 c.c. of culture and no local abscesses were produced by subcutaneous inoculation. Of 3 animals injected intravenously, one showed a small area, apparently of consolidation, near the hilum of one lung and another a more doubtful lesion in the same region. Cultures, however, were negative.

Isolation of the organisms from Swiss mice

An attempt was made to isolate pleuropneumonia-like organisms from a stock of Swiss mice, originally obtained from the Rockefeller Institute in America, by the use of the same methods. Three normal mice were killed and suspensions of their lungs inoculated intranasally into three batches of 12 mice. Typical lesions developed in the lungs of 1 mouse in one batch and of 3 in another. Passage from one lung produced lesions in 3 out of 6 mice, from another in 2 out of 6, but with the other two passage was unsuccessful. From 3 of the originally affected lungs pleuropneumonia-like organisms were isolated. It was found that one of these (strain 6), when plated on horse serum agar, produced discrete colonies which although minute could be subcultured. When observed under low magnification, the colonies had an irregular surface which made them opaque. The colonies in a growing culture were of different sizes and the smaller and younger colonies were translucent. Subcultures from the different types of colony produced the same kind of growth: there was no evidence that it was a mixed culture. The colonies were firmly adherent to the medium owing to penetration by the growth, as is characteristic of this group of organisms.

Agglutination results

An antiserum was prepared against strain 4 by repeatedly inoculating a rabbit intravenously with centrifuged deposits from rabbit serum broth cultures suspended in saline. Agglutination tests were carried out by using a modification of the technique described by Klieneberger (1938). Antigens were obtained by resuspending the centrifuged deposit from horse serum broth cultures in buffered saline containing 0.25 per cent. formalin. Tests were carried out at 50° C. and read at the end of four hours. The homologous strain was agglutinated to a titre of 1:640 and a comparable titre was obtained with strains 1, 2 and 5, all isolated from farm mice, and with strain 6, which had been recovered from the Swiss mice and had given small surface growths. These five strains would appear to form an antigenically similar group. Strains 3 and 7 were not agglutinated and are therefore antigenically distinct. Strain 7 was recovered from the Swiss mice, but has not been thoroughly investigated. Strain 3 was isolated from farm mice. It does not appear to differ in its properties from

the other strains. It can produce pneumonia when inoculated intravenously but its pathogenicity when inoculated in other ways has not yet been investigated.

Discussion

Findlay *et al* (1938, 1939-40) have described two different pleuropneumonia like organisms isolated from the brains of mice. One, the L 5 organism, has on several occasions infected mice which had been injected intracerebrally for the investigation of other infective agents. Its inoculation intracerebrally into mice produces nervous symptoms and the condition called "rolling disease". The other organism, L 6, was encountered in the brain of mice which had been inoculated with the blood of splenectomised mice. It did not produce nervous symptoms when inoculated intracerebrally, but multiplied in the brain (Klieneberger, 1939). It caused polyarthritis when injected intravenously or into the pads of the feet. Klieneberger (1935, 1936) originally described an organism of this type occurring as a symbiont of the *Streptobacillus moniliformis*, an inhabitant of the throat in mice and rats, but Dienes (1939) has recently claimed that it is a variant of that bacillus.

Sabin also has isolated pleuropneumonia like organisms from mice in America. He independently described an organism (strain A) similar to L 5. It produced marked neurological changes, but differed in being more pathogenic, in forming a toxin and in producing arthritis in mice. Another strain (B) was cultured directly from the brain of a normal mouse (Sabin, 1938b). It produced arthritis in mice following intravenous injection. Strains A and B are not serologically identical, but share a common antigen (Sabin, 1939a). More recently Sabin (1939b) has published a short note describing the isolation of these organisms by direct culture from the conjunctivæ and from the nose. In this way he recovered a third antigenically distinct strain (C) which also produced arthritis.

Sullivan and Dienes (1939) isolated pleuropneumonia like organisms directly on serum agar plates from pneumonic lesions produced by inoculating mice intranasally with normal mouse lung suspensions and making serial passages. Sabin found some of these strains antigenically similar to his strain A, but they did not give rise to arthritis nor damage the central nervous system when injected intravenously. Sabin also found that the strains encountered by Swift and Brown (1939) during their investigation of rheumatic fever were serologically identical with his strains A, B and C.

It would appear that the organisms described in this paper differ in several respects from those previously reported. They do not form a neurotoxin or produce polyarthritis and they grow but poorly on solid media. On the other hand when inoculated intravenously and intraperitoneally, they produce lesions in the lungs and histological changes in the liver.

It has already been pointed out how these findings emphasise certain dangers in using intranasal inoculation of mice for the study of viruses. When, for instance, this method is used for titrating influenza virus or for preparing a vaccine, the possibility should always be borne in mind that the stock of mice used may harbour pleuropneumonia-like organisms which may be washed into the lungs during inoculation and may there produce lesions.

Summary

1. The isolation of pleuropneumonia-like organisms from normal mice of two different stocks is described.
2. It is shown that intranasal inoculation of these organisms into mice produces pneumonia in a large percentage of animals.
3. Intraperitoneal inoculation produces lesions in the lungs and microscopical focal infiltration in the liver.
4. Details of the properties of the organisms are given and a description of the lesions produced. The histological changes in the lungs are a combination of inflammatory exudation and localised reticulum-cell hyperplasia.
5. It is emphasised that when intranasal inoculation of mice is employed for the investigation of viruses the possibility of the occurrence of lesions due to pleuropneumonia-like organisms must always be borne in mind.

I gratefully acknowledge my indebtedness to the late Sir Patrick Laidlaw and to Dr C. H. Andrewes for their help and frequent suggestions and to Dr W. J. Elford for his help and advice in the ultrafiltration analysis.

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THE VASA VASORUM OF THE FEMORAL VEIN

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THIS work was planned as part of an investigation into the causation of femoral thrombosis. Present conditions have interrupted this enquiry and the following paper deals only with results obtained in normal femoral veins, together with some observations on pathological vessels.

The most satisfactory method of studying vasa vasorum is that used by Winternitz, Thomas and LeCompte (1938) with striking success in their study of the aorta and coronary vessels. The attached femoral artery and vein were dissected out in the post-mortem room and a cannula was inserted into one end of the artery, the other end being ligated. The cannula was connected by a rubber tube to a flask containing a 1 : 8 aqueous suspension of Higgins' engrossing ink. Pressure was gradually increased in the flask to 120 mm. Hg and the ink then allowed to flow into the closed artery for fifteen minutes, after which the pressure was raised to 300 mm. and maintained at that level for $\frac{1}{4}$ hour. Injection of both arterial and venous vasa was by this time complete and the preparation was fixed in formol-saline and either cleared directly in glycerol, or in xylol and canada balsam after dehydration in alcohols. To render visible the vasa in the artery, the inner coats had to be stripped off but this was not necessary with the vein.

The vasa vasorum of the normal femoral vein

Small arterial branches, having separated from the femoral artery or its muscular branches, run in the connective tissue between the femoral artery and vein in company with venules. They enter the adventitia of the vein, where they branch and continue their course in a direction parallel with its long axis. These large paired vessels can be traced for an inch or so in proximal and distal directions from the point of entry. At intervals they give off smaller branches which penetrate into the media, where their course will be followed. The terminal branches of the larger adventitial vasa anastomose with corresponding vessels arising from adjacent paired systems (fig. 1). In this way an extensive network of large paired vessels is formed in the adventitia from which arise branches to supply the deeper layers of the media. The latter after entering the media break up to form a very characteristic plexus on the outer (medial) side of the internal elastic lamella, here referred to as the sublamellar plexus.

The components of the large paired vasa exhibit contrasting features which allow of their identification in any given field. For

convenience they are here spoken of as type 1 and type 2 vasa (fig. 2). Type 1 vasa frequently appear to curl or undulate in their course, due probably to small amounts of smooth muscle fibres in their walls. They inject more deeply than the type 2 vasa as though nearer to the head of pressure, so that the presumption is that they are of arterial origin. They never show any tendency to branch and rejoin except when they anastomose with the corresponding terminal of type 1 vasa running in the opposite direction. Type 2 vasa either accompany the type 1 vessels after the manner of *venæ comites* or they break up to form extremely complicated plexuses closely surrounding the type 1 vasa, especially where

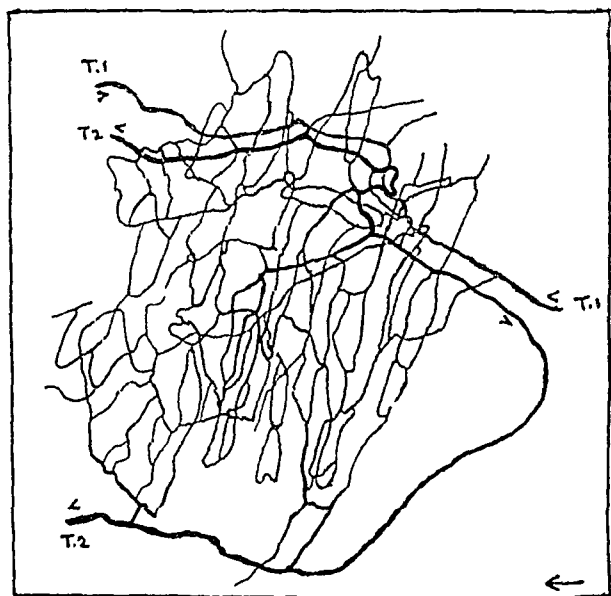


FIG. 1.—The anastomosis is shown between two pairs of neighbouring vasal systems. The loops of the sublamellar plexus are seen to lie in the transverse diameter of the vein. $\times 20$.

These drawings were made by camera lucida. The arrow in one corner of the figure indicates the longitudinal axis of the vein and direction of the venous blood-flow, the small arrows the direction of flow in the vasa vasorum. Black vessels are type 2 (efferent), red vessels type 1 (afferent), except in figs. 6 and 8, where the sublamellar plexuses are red, and the parent vasa and nodal plexuses black.

these give off a branch. For this reason I refer to them as nodal plexuses (fig. 3). They are entirely distinct from the sublamellar plexuses and are situated in the outer layers of the adventitia. Type 2 vasa are smaller in calibre than type 1 vasa and pursue a straight course with little or no undulation or curling. They are venous in nature.

The sublamellar plexus is composed of long loops whose opposite ends are derived from the paired types 1 and 2 vasa in the adventitia.

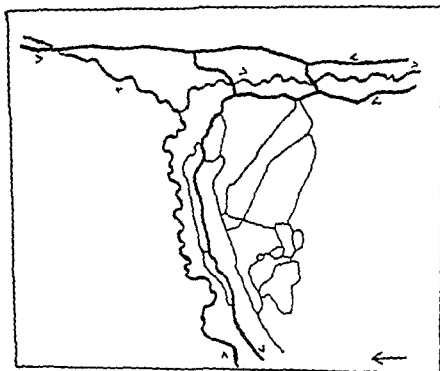


FIG 2—The curling course of the type 1 (afferent) vessel and its lack of branches contrast with the straighter but branching course of its type 2 (efferent) companion $\times 20$

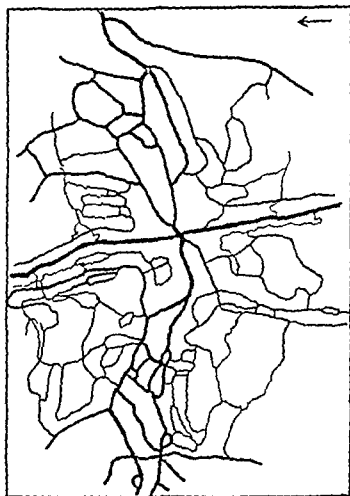


FIG 3—Typical nodal plexuses of type 2 vasa surrounding a type 1 vessel The sublamellar plexus is not shown $\times 20$

The loops thus complete the circulation from the arterial to the venous side (figs 4 and 5) Appearances frequently suggest a direct communication between types 1 and 2 vasa but careful examination

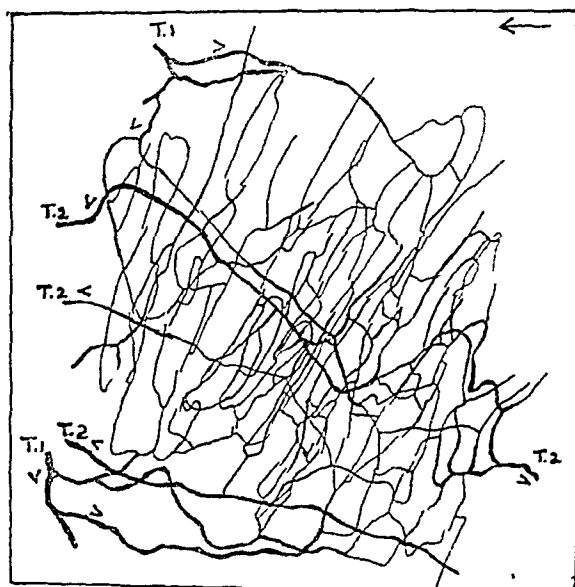


FIG. 4.—Portion of a sublamellar plexus showing capillary loops formed from types 1 and 2 parent vasa. $\times 20$.

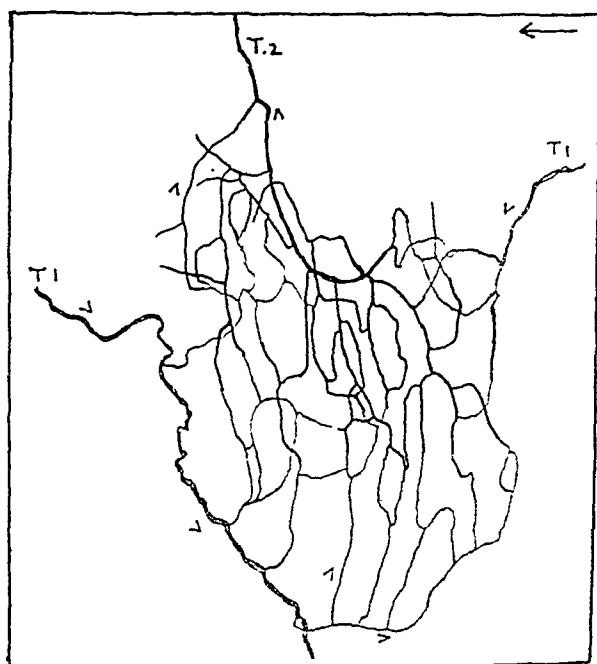


FIG. 5.—Portion of a sublamellar plexus less highly developed than the previous one (fig. 4). $\times 20$.

always reveals two vessels superimposed and merely simulating one. In the femoral vein the loops course in the transverse axis, but in

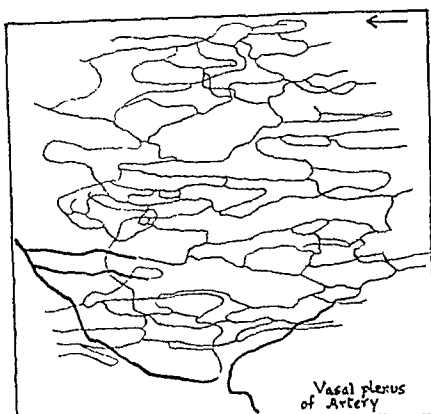


FIG. 6.—Vasal plexus of femoral artery. The capillary loops (red) lie in the longitudinal axis. The black vessels represent parent vasa. $\times 20$.

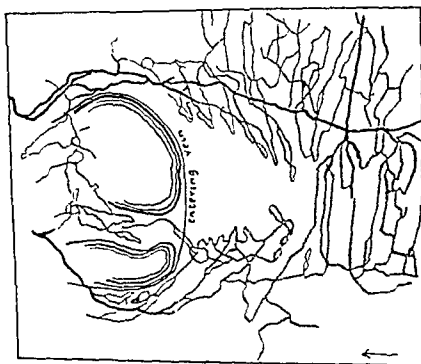


FIG. 7.—Mouth of an entering vein showing an avascular area on its distal edge. The black vessel is a large parent vessel and capillary loop formation is seen at both edges of the bare area. $\times 20$.

the femoral artery (fig. 6), which possesses an equally numerous and similar vasal pattern, the direction of the loops is longitudinal and this is a constant point of difference between artery and vein. Since the plexus is three-dimensional it cannot be accurately

represented on a flat surface, and hence in longitudinal sections of veins the vasa appear as dots, but in transverse sections, where the vasal loops lie approximately in the plane of the section, they appear as delicate threads. The innermost parts of the plexus are almost in contact with the internal elastic lamella and the outer portions lie at the junction of media and adventitia. The paired vessels from which this plexus is derived are often seen to be on a deeper level than the more superficial loops of the plexus. There is, however, no tendency on the part of the parent vasa and plexuses to penetrate the internal elastic lamella in healthy veins. I regard any such penetration as evidence of disease of the vein wall.

Development of the vasal system

The development of the vascular system in a vessel wall seems to depend on the formation of paired branches from arterioles and

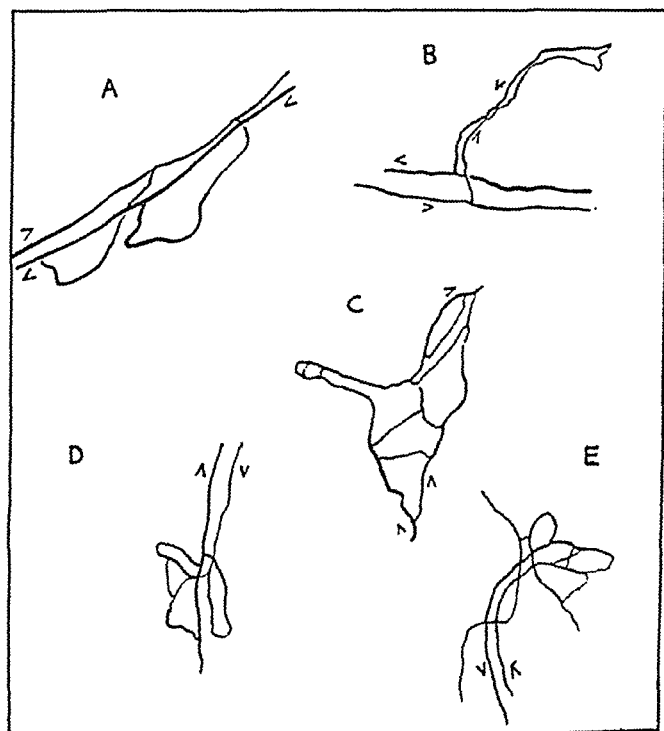


FIG. 8.—Five stages in the development and organisation of a capillary loop. In E, the loop has reached the sublamellar level and is spreading outwards. In the original specimens, the loop is seen to be considerably nearer the lamella than are the parent vessels from which it is formed. $\times 40$.

venules which penetrate as loops to the sublamellar level. Both the afferent and efferent systems are represented in this vascular unit. In the femoral vessels from a girl of 19 years the vasal system was found to be on a much more simple scale than in the adult. All

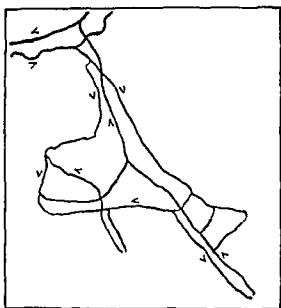


FIG. 9.

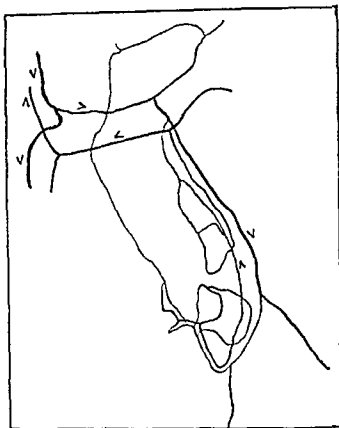


FIG. 10.

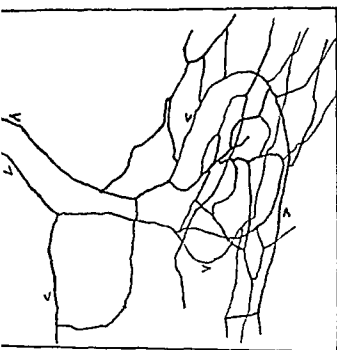


FIG. 11.

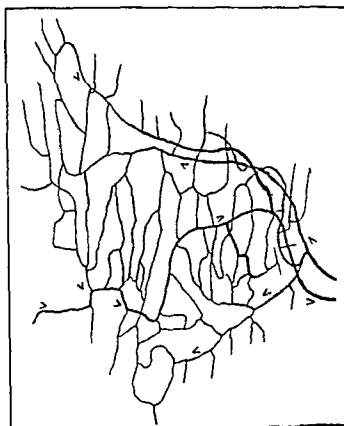


FIG. 12.

FIGS. 9-13.—Five stages in the development of a mature sublamellar plexus from capillary loops. In fig. 11 anastomosis has been established with an older portion of a neighbouring system. In fig. 12 two primary loops are still just recognisable. In fig. 13 the plexus is mature and the original loop, forming this portion of the plexus, can hardly be traced. FIGS. 9-13 $\times 20$.

stages in development could be traced from simple unbranched afferent-efferent loops to their anastomosis with neighbouring loops and finally to a well developed, though wide-meshed, sublamellar plexus (figs. 8-13). In adult veins such simple capillary loops are seldom observed, since the plexus is fully formed and close-meshed. It seems likely therefore that the sublamellar plexus is developed by an ingrowth of capillary loops from the parent adventitial vasa and that such capillary loops represent the primitive vascular units.

But there are numerous large vasa in the wall of a vein which cannot be accounted for by direct origin from the arterioles and

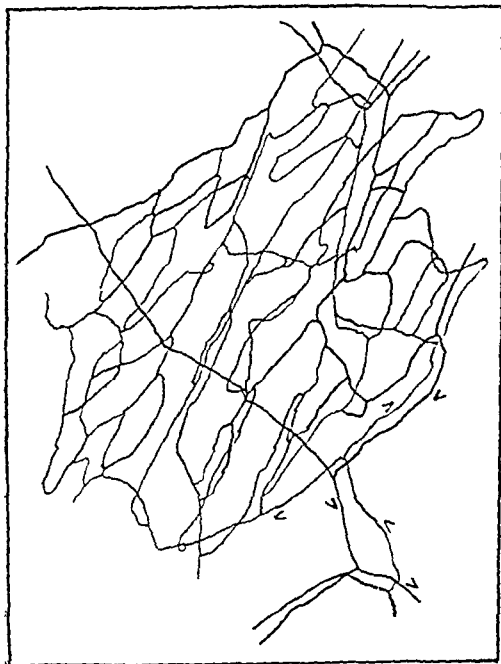


FIG. 13.

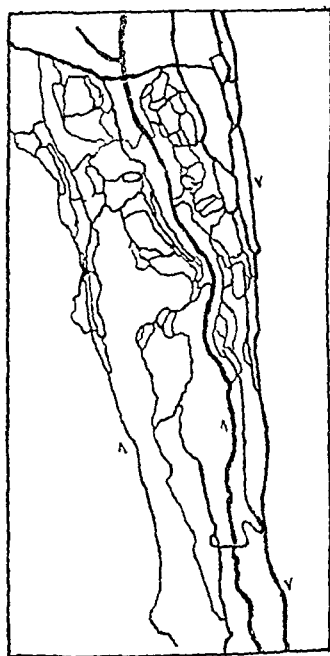


FIG. 14.—Nodal plexus showing condensation of two venous trunks (black) at the lower part of the figure. $\times 20$.

their paired branches. On the analogy of vascular primordia in the embryo, it may be assumed that a capillary network is the first structure to be laid down and that the parent vessels are derived from this network by a process of condensation and fusion of certain parts (fig. 14). Support for this view is to be seen in the nodal plexuses which occur in the adventitia in connection with the venous (type 2) vasa and lie on each side of the arterial (type 1) vasa. These plexuses are elaborately developed and in places may be seen to condense to form a double and finally a single venous

(type 2) trunk. At first sight no arterial connections seem to be present (fig. 15), but if the accompanying (type 1) arteriole is

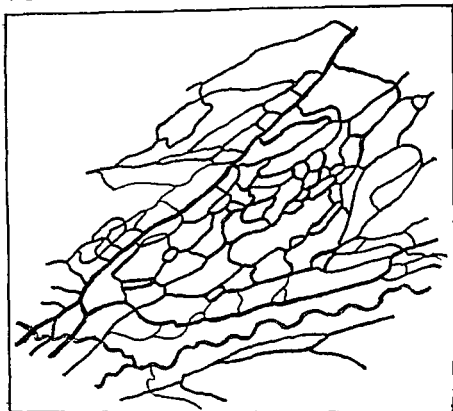


FIG. 15—Nodal plexus derived entirely from vasa of type 2. In this section of the field there are no communications with type 1 vasa $\times 20$.

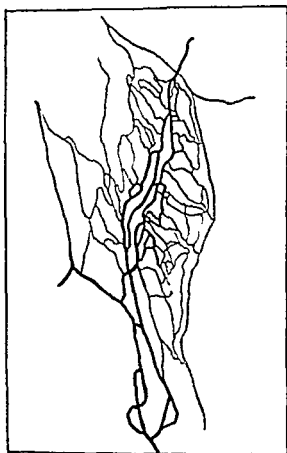


FIG. 16—Nodal plexus (black), with frequent arterial connections at its periphery, which are condensing to form a single trunk. $\times 20$.

examined minute branches are seen to spring from it and to ramify and anastomose with the nodal plexus (fig. 16), especially on the

edge of the plexus remote from the arteriole, where other minute arterioles also enter from the surrounding field. In other words the nodal plexuses are composed of loops connecting arterioles and venules, but the arteriolar connections are slight and the mass of the plexus consists of venous capillaries which condense to form larger venules and finally a single venous trunk. Nodal plexuses are most striking in young subjects but are also present, though in an attenuated form, in middle age. In all cases the plexus is chiefly venous in nature and I have not yet seen a similar plexus condensing to form arterioles. It is possible, however, that further study of vessels from young subjects might reveal traces of such arterial condensation, at a stage before the afferent side of the vasal system had assumed the characteristics of type 1 vessels.

The microscopic appearances of nodal plexuses are quite sufficient to distinguish them from the sublamellar plexuses. The former are only found in the adventitia and they give few branches to the sublamellar plexus, although in places it appears that portions of nodal plexuses are in process of sinking bodily to a deeper level. The nodal plexuses therefore do not represent a stage in the development of the sublamellar plexuses.

Vasa vasorum of phlebosclerotic veins

It will be remembered that the normal direction of capillary loops is in the transverse axis of the vein. In phlebosclerosis, unaccompanied by thrombosis, longitudinal loops are found and these are always intimal and may reach a considerable length (fig. 17). They are derived from those loops of the sublamellar plexus which lie nearest the internal elastic lamella. A localised growth of vasa is also seen, and this results in the formation of a tree-like plexus which burrows through the internal elastic lamella and invades the intima. These arborescent loops are more common in thrombosed veins where they establish communication with a system of endothelial-lined spaces which form around the original platelet scaffold in the thrombus. The intimal loops are frequently the site of hæmorrhage which may be so extensive as to surround the vein with a zone of red "infarction" situated on the intimal side of the internal elastic lamella. Such changes are more often seen when thrombosis has occurred in the femoral vein and they are frequently associated with atheroma of the accompanying femoral artery.

The vasa of thrombosed veins

Vasal invasion of the thrombus takes place at the edges of its attachment to the intima. Typically, there is ingrowth of capillaries from the sublamellar plexus, the internal elastic lamella becoming frayed and disorganised in the process, but in the case of phlebo-

sclerotic veins the arborescent and longitudinal loops grow out into the thrombus (fig 18) At the same time endothelial lined spaces, following the platelet scaffold, unite with the intimal vasa and become filled with blood The endothelium of the former is derived from that which has grown over the thrombus from the wall of the vein As a result of their anastomosis with the

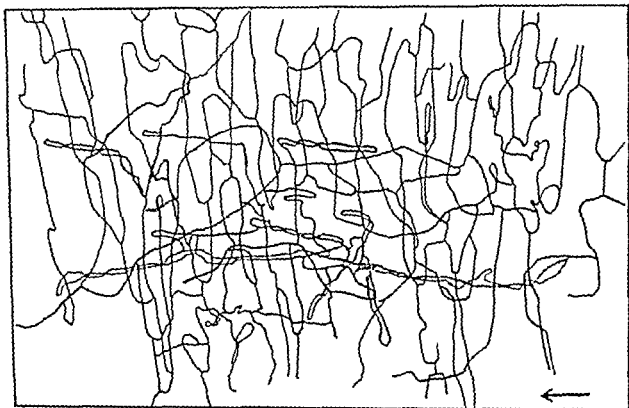


FIG 17—Longitudinal loops (red) from a case of phleboscrosis The contrasting direction of the ectopic loops with that of the normal plexus (black) is well shown $\times 20$

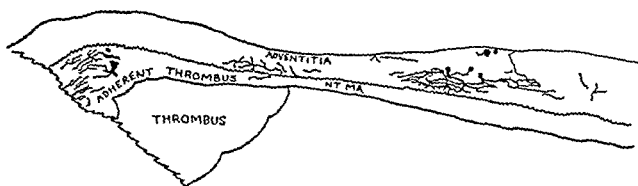


FIG 18—Transverse section of a vein with organising thrombus injected to show vasa Vasa activity is noted at the edges of the thrombus where both it and the intima are being invaded The media and adventitia of a healthy vein show an even distribution of vasa plexuses In cases of thrombosis the plexuses disappear from large areas as shown here $\times 6$

endothelial lined spaces these thrombal vasa open on the surface of the thrombus into the remains of the original lumen They are the only examples I have found of vasa which open directly into the lumen and they are always associated with thrombosis

But though vasal hypertrophy is well marked at the edges of attachment of the thrombus, there is a tendency to disappearance of the normal vasal system elsewhere in the vein wall. Two sites call for special mention. The first is at the distal edge of the mouth of an entering vein or venule (fig. 7), where a "bare area" may extend as much as $\frac{1}{8}$ inch distally along the wall of the larger vessel. In some cases long vasa connect the normal loops on each side of this area; in others no such loops exist and the area is truly avascular. Frequently, young capillary loops are seen growing from the edges of the "bare area", or the rim of the mouth of the entering vein may be completely encircled by a narrow band of plexus. In all these cases, however, the "bare area" can be made out. The second site which is relatively avascular is the sinus of a valve. The valve leaf itself never contains vasa, being derived from structures on the intimal side of the internal elastic lamella. The wall of the valve sinus frequently shows no vasal plexus and it is suggested that this is a result of pressure atrophy due to the presence of the thrombus. In the same way a long strip of vein wall in cases of thrombosis may become devascularised at points opposite the attachment of the thrombus (fig. 18).

Summary

1. The femoral vein receives its blood supply from a capillary plexus lying on the medial side of the internal elastic lamella. In the normal vein, the intima contains no vasa.

2. This sublamellar plexus is composed of capillary loops derived from paired elements in a wide network of arterial and venous branches situated in the outer layers of the media and adventitia.

3. The efferent (venous) branches in the adventitia form complicated nodal plexuses which are distributed on each side of the afferent (arterial) vasa, especially where the latter subdivide.

4. It is suggested that these nodal plexuses represent the vascular primordia from which the efferent vasa are formed by condensation and fusion.

5. In phlebosclerosis and thrombosis vasa are found in the intima and these frequently assume unusual patterns.

6. In thrombosis, a very constant feature is the disappearance of the vasal plexuses from those parts of the wall of the vein where the thrombus is not attached, from the valve sinuses and from the distal edge of the mouth of an entering vein.

7. A zone of intimal hæmorrhage is frequently associated with the invasion of the intima by vasa.

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THE CHEMOTHERAPY OF EXPERIMENTAL STAPHYLOCOCCAL INFECTION IN MICE WITH DRUGS OF THE SULPHANILAMIDE GROUP*

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AN important requisite for therapeutic investigations on infected animals is that the experimental disease should pursue a regular course, preferably with a fatal issue in a fairly narrowly defined period. With the staphylococcus in mice this is difficult to attain. Among early investigators Kuhnau (1897), who injected intraperitoneally a suspension of a 20 hour agar slope culture in 5 c.c., considered a strain highly virulent when 0.01 c.c. proved fatal and weakly virulent if 0.2 c.c. was required to cause death. von Lingelsheim (1900) noted that intramuscular injection in repeated passages reduced the fatal dose of broth cultures for rabbits from 5 c.c. to 0.01 c.c., but caused much less relative increase in virulence for the mouse. Kolle and Otto (1902) obtained irregular results in mice. Since the need has arisen for testing the sulphanilamide group of drugs on staphylococcal infections, the mouse has again been extensively used, and the results of different workers show considerable divergence.

Buttle *et al* (1936) mentioned that the strains available would kill mice only in a very large dose. Domagk (1937) injected intraperitoneally 0.5 c.c. of a broth culture diluted 1/2 and recorded the death of 11 out of 12 mice within six days. Buttle (1937/38) along with Parish employed the procedure of Miller (1934/35) for increasing the pathogenic effect of meningococci by injecting the organisms intraperitoneally along with 5 per cent mucin, 5 cocci of a strain of staphylococcus derived from bovine mastitis killed 8 out of 10 mice within 10 days and 500 cocci killed all 10 in a day. Again Buttle *et al* (1938) reported that an intraperitoneal inoculation of 10^{-9} c.c. of a culture mixed with 5 per cent mucin killed 4 out of 5 mice in 5 days, while 10^{-7} c.c. killed all 5 within one day. Digonnet (1939), using an 18 hour culture of Buttle's strain intraperitoneally with mucin, found the M.L.D. to be 5000 cocci. Mellon *et al* (1937/38) injected intravenously 0.2 c.c. of a 20 hour broth culture and killed 47 out of 54 animals, deaths occurred up to 4 weeks after inoculation.

* The substance of this paper was communicated to the Pathological Society at the meeting at Birmingham July 1939.

disulphanilamide 8 out of 22 survived ; with uliron 2 out of 22 survived ; while out of 33 untreated controls 4 survived, some dying very late (see p. 431). In this second series treatment of some animals began on the 3rd day after inoculation, when abscesses were already present in the kidneys ; of 22 mice receiving disulphanilamide 9 survived, with uliron none out of 22. It was concluded that the time at which treatment is begun is "not critical" and that the results are in marked contrast to those obtained in streptococcal infections. Feinstone *et al.* (1938) inoculated mice intravenously with two not highly virulent strains of staphylococci which in the large dose used (0.2 c.c. of a suspension of a 16-hour slope culture in 6 c.c. of broth) killed 45 out of 50 controls, 2 dying as late as the 15th day. Fifty mice were given 0.01 g. of each drug by mouth 15-30 minutes after inoculation, two similar doses daily for the first five days and one daily for the next five ; they were observed for 30 days. With sulphanilamide 17 survived, with disulphanilamide 22 and with uliron 15. They stated that these drugs are "of little value in invasive tissue infections in the mouse due to staphylococci". Whitby (1938) compared sulphanilamide and M. & B. 693 (sulphapyridine). He gave 0.02 g. doses of sulphanilamide by mouth, at the time of inoculation, 7 hours later, twice daily on the 2nd, 3rd and 4th days and once on the 5th, 7th, 8th and 9th days ; of M. & B. 693 (2-(*p*-aminobenzene sulphonamido pyridine) or 2-sulphanilyl amino pyridine), 0.04 g. doses were given at the same times. Of two groups of 6 mice inoculated respectively with 15,000 and 1,500,000 cocci, 4 and 2 mice survived for 28 days after treatment with the former drug, and with the latter 6 and 0 mice, 5 of the last group dying after 15-18 days : the average survival periods of the treated groups were respectively 20, 10, 28 and 14 days. The two sets of 6 untreated controls all died within one day. With doses of 15,000 and 1,500,000 cocci and of 0.01 and 0.02 g. of sulphanilamide the average survival periods in days ("maximum 28") were 22, 15, 20 and 13 respectively ; with 0.02 or 0.04 g. doses of M. & B. 693 they were 26, 17, 26 and 16 days (the numbers of mice are not given). All the controls lived less than one day. Mayer (1938) injected intraperitoneally in mucin 1 c.c. of a 1 : 10 dilution of a culture which had been repeatedly passed through mice. Of 15 controls 1 survived 20 days and the average life was 1.73 days : 15 treated with sulphanilamide had an average life of 10 days and 4 survived ; of 15 treated with M. & B. 693, 11 survived and the average life was 17.7 days. With both drugs two doses of 0.02 g. were given by mouth ; the interval is not stated. Nitti *et al.* (1938) tested diamino diphenyl sulphone and diacylated derivatives ; the free diamino compound and the formyl and propionyl derivatives had marked action, while the acetyl and butyryl were less effective (dosage not specified). Buttle *et al.* (1938) compared sulphanilamide and uliron, using groups of 5 mice inoculated intraperitoneally with a series of doses of staphylococci in mucin and treated by mouth daily for 6 days with two doses of 0.025 g. and then once daily with the same dose up to 14 days. With inocula of 10^{-7} and 10^{-5} c.c. 3 mice of each group treated with sulphanilamide survived over 12 days ; in the uliron series with 10^{-5} c.c. 3 mice died in 9 days, while with 10^{-7} c.c. 3 survived over 12 days. Of 5 controls receiving the smaller inoculum, all died within one day ; of 5 receiving one-hundredth of the smaller inoculum only 1 survived over 5 days. The statement was made that in staphylococcal infections no compound has been found more effective than sulphanilamide. Digonnet (1939) inoculated mice intraperitoneally with a culture (obtained from Buttle) along with mucin ; sulphanilamide (0.01 g. by mouth for 3 consecutive days) protected against 1000 lethal doses (six million cocci), but was not certain, as after 10-15 days staphylococci might be found in the organs and often death

occurred late with milary abscesses. Similar treatment with ultron was less effective, and prontosil (sulphamidochrysoidin) had only weak action, M & B 693, as compared with sulphanilamide, was of equal or greater effect. *Diformyl diamino diphenyl sulphone* given in the same way was active, but the diacetyl analogue (rodilone) was much less so. Powell and Chen (1939) inoculated 2 sets of 5 mice intraperitoneally with doses of 10^{-3} and 10^{-4} c.c. of a culture diluted with 0.5 c.c. of 5 per cent mucin and one hour later gave by mouth a dose of 0.03 g. of M & B 693, which was repeated 8 hours later and once daily for the next three days. "A curative action" is described, as 1 out of 5 treated mice which had received the larger inoculum survived over 14 days and all of 5 which received the smaller. In 3 sets of 5 controls receiving respectively inocula of 10^{-3} , 10^{-4} and 10^{-5} c.c., all died except 1, while with a dose of 10^{-4} c.c. none died. Sulphanilamide, it was stated, "frequently prolonged life but seldom saved it". Bliss and Long (1939), following the same scheme as Feinstone et al., found that out of 49 mice treated with M & B 693, 16 survived for 15 days, and with sulphanilamide 4 out of 50 while of 30 controls none survived beyond 6 days. They concluded that "the chemotherapeutic effect of sulfapyridine in staphylococcal infections in mice is definite enough to warrant careful clinical trials."

The results show contradictions and frequently the number of animals used has been small and the period of observation short. Also, the dosage of the inoculum and recorded course of the infections are variable. Some workers have observed irregularities similar to those which we have experienced, while according to others the staphylococcus may kill mice almost as regularly as virulent streptococci. Curative effects have been repeatedly recorded, but there is a great measure of agreement that staphylococcal infections do not respond to the sulphanilamide group of drugs so well as the streptococcal.

The clinical importance of the problem of staphylococcal infections made it desirable to investigate further certain of the more effective compounds of the group as judged by their action on streptococci in mice. Among them especially are monoacetyl diamino diphenyl sulphone* and M & B 693, promising results having already been recorded by others with the latter. These are eminently "depot" compounds which are slowly absorbed from the tissues. For comparison the effect of members of the group which are rapidly absorbed and excreted, such as sulphanilamide, prontosil soluble and related dyes, was also tested.

The mice were inoculated intraperitoneally with 0.5 or 1.0 c.c. of a dilution in 5 per cent mucin of a 20-24 hour broth culture of one strain of *Staphylococcus aureus*, the dilutions ranged from 1:5 to 1:500 and were estimated, according to the behaviour in preceding passages, to kill a considerable proportion of untreated animals within two or three days by septicæmia. During the whole period of the experiments the strain was alternately passed through mice and cultured in broth. Of 859 mice

* This compound was prepared by Imperial Chemical Industries Ltd., for its action on streptococcal infections of mice see C. H. Browning (1939).

inoculated in the course of these passages, 68 were alive at the end of 2 weeks (7.9 per cent.), while the rest lived on an average 1.7 days. Of these, 90 mice constituted the controls for the 11 therapeutic series, treated and untreated animals receiving the same inoculum in each experiment; 9 of them (10 per cent.) were alive at the end of 2 weeks, while the remaining 81 lived on an average 1.7 days. Accordingly, the course of the infection in the therapeutic controls was closely similar to that in the large series. For the therapeutic experiments the period of observation was extended to 3 weeks with the result that the number of survivors was reduced and the average duration of life of the rest slightly increased (table II).

TABLE II
Results of treatment of staphylococcal infections in mice

Treatment	No. of mice	No. of deaths	Average length of life of those dying within 3 weeks (days)	Survivors (3 weeks)	
				No.	Per cent.
Untreated controls.	90	87	2.8	3	3.3
Series comprising sulphanilamide, prontosil-soluble and related dyes (1:100, 1:150 × 2) *	89	85	2.6	4	4.5
Uliron (1:100, 1:150 × 2) . . .	10	10	2.1	0	0.0
M. & B. 693 (1:150, 1:200 × 2) .	23	18	4.8	5	22.0
Monoacetyl diamino diphenyl sulphone (1:150, 1:250 × 2—1:300, 1:450 × 2)	88	71	5.3	17	19.3
Ditto + antitoxin 18 hours before inoculation	33	20	6.0	13	39.0
Antitoxin 18 hours before inoculation	25	20	8.2	5	20.0

* With one slightly more toxic dye given to 6 mice, the dosage was 1:200, 1:400 × 2. For explanation of dosage see text below.

The scheme of treatment was to give one subcutaneous injection at the time of inoculation, followed by a second 4-5 hours later and repeated after 24 hours. The dosage was reckoned on the basis of mice weighing 20 g.; thus in table II 1:100, 1:150 × 2, means a dose of 0.01 g. at the time of inoculation and one of 0.0066 g. after 4-5 hours and repeated after 24 hours. With the relatively insoluble compounds—M. & B. 693, monoacetyl diamino diphenyl sulphone and uliron—toxicity is difficult to estimate accurately, but the dosages used, both of these and of the soluble drugs—sulphanilamide, prontosil-soluble and related dyes—were known to be well borne although high. The subcutaneous route was selected in order to avoid irregularities depending on varying rates of intestinal movement, etc. Of the compounds of low solubility fine suspensions were used.

Table II summarises the series of eleven experiments. In each at least one of the more effective compounds and one of the less effective were included. The results are on a sufficient scale to indicate definite therapeutic activity of the monoacetyl diamino diphenyl sulphone compound, which was tested in every experiment, while they also confirm those of others on the action of M. & B. 693 and the relative ineffectiveness of sulphanilamide, the prontosil dyes and uliron.

A frequent observation was that in spite of treatment which prolonged life or effected cure, mice became seriously ill within a few hours, just like those untreated, and remained so for several days, further, the ultimate result was not related to the early onset of illness. It is, of course, to be remembered that the pathogenic action of staphylococci may depend on their possessing several variable properties, as is emphasised by Blair (1939) in his review. Therefore therapeutic effects may be produced by agents operating together to counteract one or other of such properties. Accordingly in several groups a dose of 160 international units of staphylococcus antitoxin (product of the Wellcome Laboratories) was injected subcutaneously on the day before inoculation and drug treatment. While the numbers are small, they suggest strongly the value of combined drug and antiserum treatment, they also indicate that under the conditions observed antitoxin itself had definite prophylactic action. A striking feature was that the animals which had received antitoxin, alone or in combination with the drug, looked well during the first few days after inoculation.

Summary

1 In confirmation of earlier observations, *Staphylococcus aureus* from human lesions is in general not highly pathogenic for mice. Adult mice of similar weight vary greatly in their behaviour on intraperitoneal inoculation with staphylococci even when mucin is added to the inoculum. It has not been possible to raise the virulence of the strain extensively studied to a fairly constant high level in spite of over 200 cultivations in broth alternated with animal passages. Hence considerable numbers of therapeutic experiments are required to exclude chance results.

2 It has been shown for the strain investigated that mono acetyl diamino diphenyl sulphone has a definite therapeutic effect, prolonging the average duration of life of animals which ultimately die and increasing the number of survivors as compared with the untreated controls. Confirmation has been obtained of the activity of M & B 693 in these respects and of the relatively weak therapeutic effect of sulphanilamide, the prontosil group of dyes and uliron.

3 It is shown that staphylococcal antitoxin when given prophylactically has itself some protective action and that it may increase the curative effect of a drug.

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PASSIVE PROTECTION OF MICE WITH IMMUNE
HORSE SERUM AGAINST LIVING STAPHY-
LOCOCCI

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LITTLE doubt now remains that the administration of serum containing staphylococcus antitoxin will delay or prevent fatalities from staphylococcal infection in human and experimental subjects; reviews by Rigdon (1937) and by Bigger (1937) give a number of references which provide adequate evidence. Such antitoxic sera are usually assayed in terms of the international standard unit of antitoxin, which is based on the hæmolytic, dermonecrotic and lethal action of staphylococcus toxin upon laboratory animals (Hartley and Smith, 1935). For routine work, hæmolysis of rabbit erythrocytes commonly suffices as a means of titrating serum, and since 1938 (Report of the Permanent Commission on Biological Standardization, 1939) the result has been expressed in units of standard staphylococcus α antitoxin. There is also evidence that active immunity may be induced by the administration of toxoid, and that on the whole the level of immunity reached may be estimated by the α antitoxin content of the serum (Kitching and Farrell, 1936; Rigdon, 1937; Bigger, 1937; Smith, 1937); unfortunately, however, the correlation is not perfect. Irrespective of any controversy concerning the complete identity of the three toxic factors used in assays, it should be observed that the standard unit is not based upon measurements of other potentially pathogenic factors such as leucocidin, plasma coagulase or enterotoxin. The protective effect of serum or the immunity induced by injection of toxoid might conceivably be due to the presence of other antibodies, perhaps including those deriving from somatic antigens. Accordingly, horses were immunised with monovalent washed formalinised vaccines and monovalent and polyvalent toxoids and toxins, and their sera injected into mice prior to infection with living staphylococci. The present report presents the results obtained in these tests, and attempts to evaluate the relation between α antitoxin level expressed in international standard units and protective power.

MATERIAL AND METHODS

Immunisation of horses

In three of the horses used (655, 656, 735) the initial titre of the serum was 0.8 unit of α antitoxin per c.c., while in the fourth (651) it was 25 units.

Horse 655. From 15.6.36 till 21.9.36, except for 5 weeks in July and August, the animal received weekly subcutaneous doses of washed Wood 46 formalinised vaccine (Kitching and Farrell), rising from 5 c.c. to 100 c.c. Beginning with 10 c.c. on 29.9.36, injections of the same antigen were given intravenously till the sample for protection tests was taken on 2.11.36. During immunisation the α antitoxin level rose only slightly, the maximum titre being 25 units and that at the time of bleeding 7.5 units per c.c. The agglutinating titre for Wood 46 rose from 250 to 5000 within one month of the commencement of injections but had declined to 1300 by 2.11.36. The antileucocidin titre was 0.18 K (Valentine, 1936). This serum is designated hereafter as "antibacterial Wood 46."

Horse 656. From 15.6.36 till 29.9.36, except for five weeks as above, this horse received weekly injections of Wood 46 alum toxoid (Farrell and Kitching, 1938) and toxin—5.50 c.c. of alum toxoid and 5.120 c.c. of toxin. On 2.11.36, when the sample for protection tests was taken, the α antitoxin titre was 110 units, while the agglutinating titre for the homologous organism had risen from 125 to 500. The antileucocidin titre was 1.25 K. This serum is designated hereafter as "antitoxic Wood 46."

Horse 735. From 15.6.37 till 14.12.37 injections of washed formalinised 24 m.a. vaccine were given twice weekly by the subcutaneous and intravenous routes alternately in doses increasing from 10 c.c. to 225 c.c., then decreasing to 50 and 100 c.c. During this interval the α antitoxin level rose to 130 units, but at the time of bleeding for protection tests (6.10.37) it had declined to 55 units. On and after 14.12.37 a "young culture" vaccine (Lyons, 1937), made afresh every two weeks, was given weekly in doses of 200 c.c. intravenously until 22.3.38. During this period, the α antitoxin level declined gradually to 10 units, though on 14.2.38, when the horse was bled for protection tests, the titre was 16.5 units. From 30.3.38 the concentration of this "young culture" vaccine was doubled and 175 c.c. were injected intravenously each week till 4.5.38, and blood for protection tests was taken on 12.5.38 (7.5 units per c.c.). On 9.6.38, injections of 24 m.a. alum toxoid were begun and after two doses (5 c.c. and 10 c.c.) the titre had risen to 280 units on 27.6.38, when another bleeding was taken. The serum from this horse is hereafter designated "anti-24 m.a.", followed by the date of bleeding.

Horse 651. This horse was one of a group undergoing routine immunisation with polyvalent toxoid and toxin. The doses of toxoid used were 1-200 c.c. and of toxin 5-700 c.c. Injections were given weekly from 15.6.36 until 24.2.37. During this period the α antitoxin titre increased from 25 to 280 units with an average level of about 150 units. At the time of bleeding for protection tests (2.11.36), it was 170 units per c.c. The antileucocidin titre was 1.7 K. This serum is hereafter designated "antitoxic polyvalent."

Lethal test suspension

The overnight growth on an agar slope was suspended in broth and diluted with broth to correspond with a standard suspension on which plate counts had been made. On numerous occasions plate counts were made on suspensions so prepared and the number of colonies from a standard

test suspension was found to be between 300 and 700 million per c.c. A volume of 0.5 c.c. was injected intraperitoneally; for the strains tested, this represented about 5-10 average lethal doses (A.L.D.). Observations were made continuously for 15-18 hours and at frequent intervals thereafter.

Rate of absorption and excretion of α antitoxin administered to mice subcutaneously and intraperitoneally

To determine which route of injection of serum should be used for immunisation tests, 80 mice each weighing approximately 20 g. were divided into two groups of 40. One group received 0.6 c.c. of antitoxic polyvalent serum (=100 units) subcutaneously and the other the same dose intraperitoneally. At intervals thereafter 4 mice from each group were killed, the serum pooled and the α antitoxin titre determined. The data are summarised in table I.

TABLE I

Circulating staphylococcus α antitoxin in mice following intraperitoneal and subcutaneous injection of immune horse serum

Route of injection	Standard units of antitoxin per c.c. of pooled serum from 4 mice at									
	1 hr.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.	1 week	2 weeks	3 weeks
Intraperitoneal	24	50	55	45	38	34	15	1.8	0.2	<0.1
Subcutaneous	1.2	10	25	34	37	26	...	0.8	0.2	...

Dose = 0.6 c.c. (100 units) of antitoxic polyvalent serum.

It will be seen that the highest level was found about 12 hours after intraperitoneal injection, and about 18-24 hours after subcutaneous. This test has been repeated more or less fully several times and results within 10-15 per cent. of these have been regularly obtained. Moreover, the proportional decrease was found to be similar whether 100 or 3.5 units were injected subcutaneously. Subcutaneous injection 18-20 hours before testing with living culture gave a fair concentration of circulating α antitoxin and at the same time obviated the possible complication of local immunity in the peritoneum, where the living culture was to be injected. This method was therefore used for all serum injections reported here.

PROTECTION WITH ANTIBACTERIAL WOOD 46 SERUM

Protection with a dose of antibacterial or antitoxic serum containing 5 units of α antitoxin

Sixty mice were divided into 3 groups of 20. One group received 0.65 c.c. of antibacterial Wood 46 serum, containing only 5 units of α antitoxin, the second 0.9 c.c. of 1:20 antitoxic Wood 46 serum (0.045 c.c.), also containing 5 units of α antitoxin, while the third received no serum. In each group 10 mice were inoculated intraperitoneally with a killing dose (0.5 c.c. of a half-standard test suspension) of homologous (Wood 46) and 10 with a similar dose of heterologous (24 m.a.) staphylococci. The mean survival time of

the controls was 4.42 ± 0.09 * and 4.24 ± 0.22 hours, respectively. Of the mice receiving antitoxic Wood 46 serum, all 10 tested with Wood 46 culture had a mean survival time of 7.38 ± 0.56 hours, and 9 of 10 tested with 24 m.a. strain a mean of 6.11 ± 0.27 hours,† while the one survivor succumbed in 4 days. Of those which received antibacterial Wood 46 serum and Wood 46 culture, 4 died in 24 hours and 4 survived till the experiment was terminated at one week (mean = 91.64 ± 22.2); with 24 m.a. culture, 7 died in 24 hours and 3 survived for the week (mean = 54.45 ± 23.4).

The data from this test show that a dose of antitoxic Wood 46 serum containing 5 units of α antitoxin gave statistically significant but practically unimportant protection against both test suspensions. On the other hand, a dose of antibacterial Wood 46 serum, also containing 5 units of α antitoxin, gave statistically significant and practically important protection against the Wood 46 culture, though not against culture 24 m.a. This suggests that α antitoxin *per se* was not the sole factor involved in this protection against living culture.

Protection with varying amounts of antibacterial and antitoxic sera

Four hundred mice were divided into 20 groups of 20. Two groups received no serum. Four others received antibacterial Wood 46 serum, two of them 0.5 and two 5.0 units of α antitoxin respectively. Seven groups were given antitoxic Wood 46 serum, two 0.5, two 5.0, one 50 and two 100 units of α antitoxin. Seven groups received antitoxic polyvalent serum in doses having the same unitage as those of antitoxic Wood 46 serum. Strains Wood 46 and 24 m.a. were used as above for the lethal test suspensions.

The results of this test are shown graphically in fig. 1.

In this test, 90 per cent. of the controls receiving Wood 46 culture had a mean survival time of 3.99 ± 0.12 hours. Hence this was a rigorous test, in spite of the survival of 2 animals throughout. One of the controls receiving culture 24 m.a. also survived throughout, while 19 had a mean survival time of 4.91 ± 0.34 hours.

Here again antibacterial Wood 46 serum had more protective power against Wood 46 culture per unit of α antitoxin than antitoxic Wood 46 serum. Apparently it was necessary to inject antitoxic Wood 46 serum in doses containing 50-100 units to give as much protection as a dose of antibacterial Wood 46 serum containing 5 units. Antitoxic Wood 46 serum did not afford significantly more protection against Wood 46 culture than antitoxic polyvalent serum except at the 100 unit level ($D/SD = 3.08$).

* Standard deviation is used throughout this report as the measurement of variation.

† Animals are excluded from consideration only where the individual deviation from the mean of the whole group is 3 or more times the standard deviation of the whole group.

With strain 24 m.a. antibacterial Wood 46 serum did not show significant protection. The dose of antitoxic Wood 46 serum, containing 100 units of α antitoxin, gave much the same protection

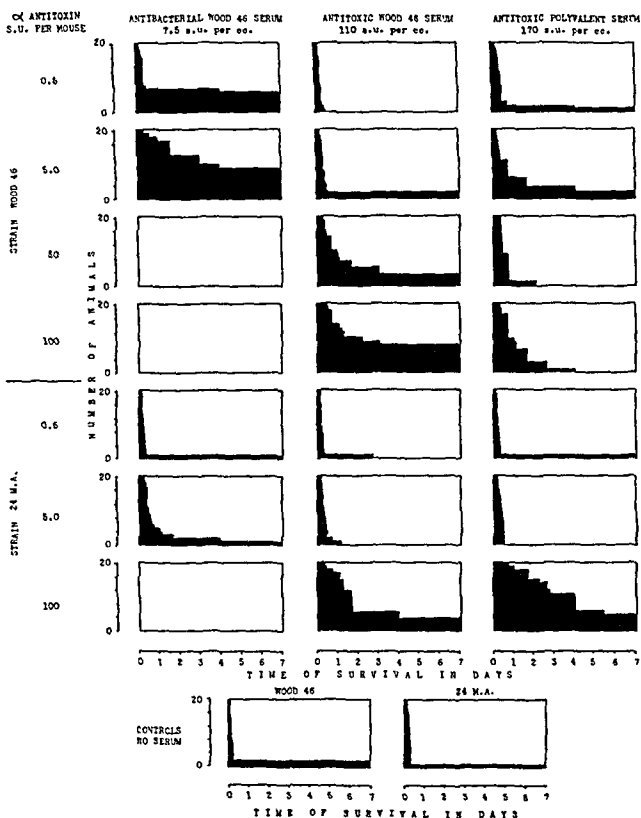


FIG. 1.—Protection with varying amounts of α antitoxin.

Blank rectangles = no animals tested. In all figs. ordinates = number of animals surviving.

as against Wood 46 culture, and the increased protection by the dose of antitoxic polyvalent serum containing 100 units was not statistically significant.

Since the α antitoxin titre of these sera appeared to be a poor index to their protective power against Wood 46 culture, attention was turned to other known antibodies—agglutinin and anti-leucocidin.

Protection following removal of agglutinin

Antibacterial Wood 46 serum had an agglutinating titre of 1300 as compared with 400 for the antitoxic Wood 46 serum. An experiment was therefore undertaken to test the protective power of the former after agglutinin had been removed by absorption with homologous and heterologous cultures. The results of this test are shown in fig. 2.

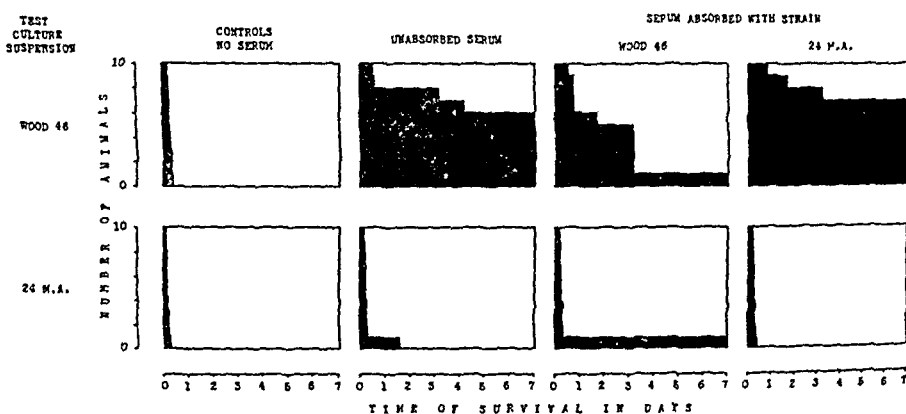


FIG. 2.—Protection with absorbed antibacterial Wood 46 serum (5 units α antitoxin).

Five 40-ounce bottles containing nutrient agar were seeded with Wood 46 culture and five with 24 m.a. and incubated at 37° C. overnight. The growth of each strain was removed with saline and heated at 56° C. for $\frac{1}{2}$ hour. After twice washing and centrifuging, the resulting volume was approximately 5 c.c. This quantity of packed cells was used for absorption of 30 c.c. of serum by heating for 3 hours at 45° C. with very frequent shaking. The material was then centrifuged and the supernatant filtered through a Seitz E.K. filter. After this treatment, agglutination did not occur with either strain in a 1 : 10 dilution of serum or higher, whether Wood 46 or 24 m.a. culture had been used as absorbent. The antitoxin titre was apparently unchanged. The absorbed and unabsorbed sera were used in mouse protection tests at a level of 5 units of antitoxin against Wood 46 and 24 m.a. living culture, in a dosage of 0.5 c.c. of a half-standard suspension, ten mice being used in each group.

The mean survival time of the control animals receiving Wood 46 culture was 5.33 ± 0.28 hours, of those receiving 24 m.a. culture 4.19 ± 0.08 hours. As before, the unabsorbed serum gave significant protection against the Wood 46 strain, 60 per cent. of the animals surviving throughout the 7-day test period, and 80 per cent. for

3 days (mean survival time = 120.7 ± 19.7 hours). After absorption with strain Wood 46 some protection remained although 90 per cent. of the animals were dead in 3 days (mean = 57.4 ± 14.3 hours). When the serum was absorbed with strain 24 m.a., which also removed the agglutinin, protection was still as great as with the unabsorbed serum, 70 per cent. of the animals surviving throughout the test period (mean = 130.9 ± 18.3 hours). No significant protection was evidenced against strain 24 m.a., though this might be attributed to the stringency of the test.

This test would suggest that the protective power of antibacterial Wood 46 serum was not dependent upon the agglutinin, which was present in high dilution prior to absorption. It also suggests that the protective factor may be reduced in some degree by absorption with homologous culture, but not with this heterologous strain under these experimental conditions. The conclusion that there was type specificity is, however, not warranted, since the proportions used for absorption may not have been optimal.

Protection and antileucocidin titre

We are very much indebted to Dr F. C. O. Valentine for being so kind as to determine for us the antileucocidin titre of these sera. He has said that the value for the antibacterial Wood 46 serum (0.18 K) is within normal limits, since a few normal horses have shown a titre as high as 0.2 K. In fig. 1, a dose of antibacterial Wood 46 serum containing 0.5 unit of α antitoxin gave significant protection against Wood 46 strain (D/SD = 3.45). This volume of serum (0.065 c.c.) is equivalent to 0.012 K. When a dose of antitoxic Wood 46 serum (0.045 c.c.) or of antitoxic polyvalent serum (0.03 c.c.) containing 5 units was used, the amount of antileucocidin administered was 0.056 K or 0.051 K, which is more than 4 times as much. Yet the protection was either definitely less or no greater. The protection of mice by the antibacterial Wood 46 serum therefore does not seem to be closely related to the content of antileucocidin.

Protection against six different strains of staphylococci

Although the experiments described so far do not prove strain specificity in the protection by these sera, it seemed worth while to test several other strains. The following experiment was carried out on 3 occasions, using quarter-standard, half-standard and standard test suspensions.

Four hundred and eighty mice were divided into 4 groups of 120 for serum injection. One group received 1.0 c.c. (7.5 units of α antitoxin) of antibacterial Wood 46 serum, one 0.9 c.c. (100 units) of antitoxic Wood 46 serum, one 0.6 c.c. (100 units) of antitoxic polyvalent serum, while the fourth,

as controls, received no serum. Each group was divided into 6 sub-groups of 20, one each for injection of the 6 living suspensions. In order to summarise these 3 experiments, the number of A.L.D. has been estimated and the results are tabulated in fig. 3.

When Wood 46 culture was used and the controls had a mean survival time of 4.77 ± 0.18 hours, significantly more protection was obtained with 1 c.c. of antibacterial Wood 46 serum (mean = 56 ± 10 hours) or with 0.9 c.c. of antitoxic Wood 46 serum (mean = 60.6 ± 12.9 hours) than with 0.6 c.c. of antitoxic polyvalent

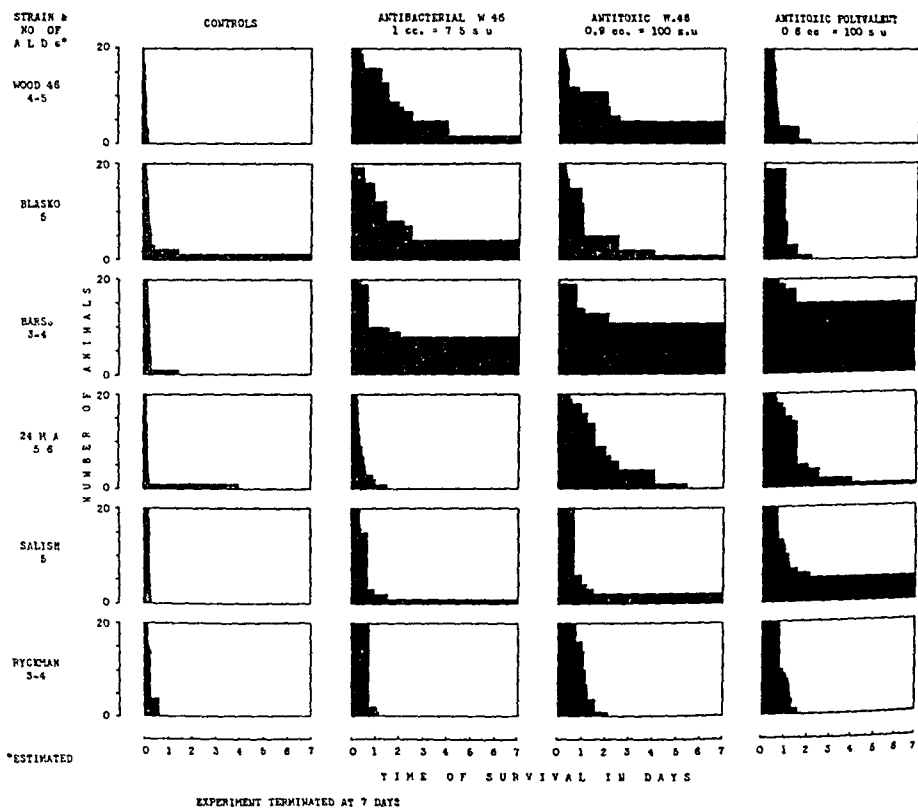


FIG. 3.—Protection with antibacterial and antitoxic sera against different strains.

serum (mean = 17.5 ± 2.4 hours). With Blasko strain, where 19 of 20 controls had a mean survival time of 6.8 ± 1.4 hours, the protection with antibacterial Wood 46 (mean = 61.8 ± 12.2 hours), but not with antitoxic Wood 46 serum (mean = 30 ± 5.0 hours), was significantly better than that with antitoxic polyvalent serum (mean = 25.6 ± 1.7 hours). With Barss strain (mean of 19 controls = 6.3 ± 0.2 hours), all sera gave some protection and, while one can scarcely say that the sera differ significantly amongst themselves, the difference in mean survival time between groups

protected with antitoxic polyvalent serum (mean = 133.4 ± 13.4 hours) and with antibacterial Wood 46 serum (mean = 79 ± 16.3 hours) is 2.45 times its standard deviation. According to Fisher's table of t , this would happen by chance less than once in 50 times. The group receiving antitoxic Wood 46 serum had a mean survival time of 108 ± 16.3 hours.

With the other 3 strains, the increase in survival time following infection was statistically significant in the group receiving antibacterial Wood 46 serum as compared with the controls. Since 90 per cent. of the animals were dead within 36 hours, however, this prolongation of life appears to be of little practical importance. The protection against 24 m.a. strain (mean of 19 controls = 3.97 ± 0.14 hours) with antitoxic Wood 46 serum (mean = 50 ± 6.8 hours) and with antitoxic polyvalent serum (mean of 19 = 37.5 ± 4.2 hours) was highly significant when these groups were compared with that receiving antibacterial Wood 46 serum (mean = 11.6 ± 1.6 hours), but it was not significantly different when they were compared with each other.

Against Salish strain (mean of controls = 4.68 ± 0.14 hours), the two antitoxic sera (mean of Wood 46 group = 33.6 ± 10.1 ; of polyvalent group = 59.4 ± 14.1 hours) were scarcely more protective than the antibacterial Wood 46 serum (mean = 15.6 ± 1.4 hours), while against Ryckman strain (mean of controls = 8.9 ± 0.6 hours) the increased protection with antitoxic Wood 46 serum (mean = 27.6 ± 1.7 hours) was statistically significant as compared with antibacterial Wood 46 (mean = 18.9 ± 0.6 hours) but scarcely so as compared with antitoxic polyvalent serum (mean = 21.7 ± 1.1 hours).

From the results presented in fig. 3, one could group these 6 strains into 3 pairs. Antibacterial Wood 46 serum was as effective as, or more effective than, the antitoxic sera against Wood 46 and Blasko strains. Antibacterial Wood 46 serum was less effective than the antitoxic sera against Barss and 24 m.a. strains. Against Salish and Ryckman strains none of these sera gave useful protection, although the trend was in favour of the antitoxic sera.

In searching for an explanation of these differences, attention naturally turned to the *in-vitro* toxin production by these strains. For comparison, the data available are presented in table II.

The *in-vitro* production of toxin, as measured by haemolysis, affords no explanation of the varying protection against these strains by these sera. Wood 46 strain produces more toxin *in vitro* than any strain except 24 m.a. and yet antibacterial Wood 46 serum afforded more protection against it than a volume of antitoxic polyvalent serum containing almost 15 times as much *a* antitoxin

and almost 6 times as much antileucocidin. On the other hand, Ryckman strain produces less toxin *in vitro* than any other strain used, but none of the sera saved the life of any mice infected with it.

TABLE II

Toxin production by six strains of staphylococci in vitro

Strain	Hæmolysin (units)		Lh dose (c.c.)
	Rabbit cells	Sheep cells	
Wood 46 . . .	2000	300	0.021
Blasko . . .	1800	250	0.20
Barss . . .	2000	2000	0.30
24 m.a. . . .	3000	4000	0.015
Salish . . .	600	100	0.15
Ryckman . . .	350	30	0.35

Units = reciprocal of dilution showing 50 per cent. hæmolysis of a 1 per cent. suspension of erythrocytes after 1 hr. at room temperature and 1 hr. at 37° C. Lh dose : see Hartley and Smith (1935).

PROTECTION WITH ANTI-24 M.A. SERA

As the data presented hitherto were suggestive of the importance of strain-specificity, the immunisation of a horse with strain 24 m.a. vaccine was undertaken. Preliminary tests indicated that serum from this horse protected against the homologous strain but not against Wood 46. One such experiment is presented here.

Sixty normal white mice were divided into three groups of 20. One group received 1 c.c. of antibacterial 24 m.a. serum of 14.2.38, a second 0.3 c.c. of the same serum diluted to 1.0 c.c. with saline, while the third received no serum. Each group was subdivided into two sub-groups of 10 for test with strains Wood 46 and 24 m.a. respectively.

The dose of Wood 46 used was about 1 A.L.D., since 40 per cent. of the animals survived throughout the 7-day test period. Any protective power which this serum possessed against this strain should therefore have been apparent. Yet only one mouse receiving 0.3 c.c. of serum and none receiving 1 c.c. survived. When the homologous strain 24 m.a. was used in such a dose that the mean survival time of 90 per cent. of the controls was 5.37 ± 0.06 hours, 70 per cent. of the animals receiving 1 c.c. of the serum survived for more than 2 days, although 80 per cent. were dead within a week. The protection by 0.3 c.c. of serum was not significant.

Since less than 1 c.c. of serum from this horse seemed unlikely to give much protection, this volume was used for a comparative test of bleedings throughout the course of immunisation, irrespective of the unitage of a antitoxin. Two hundred and ten mice were divided into 7 groups of 30

for serum injection Group 1 received no serum, group 2 the serum before immunisation was begun (15.6.37), group 3 serum after 4 months' immunisation with "old culture" vaccine (6.10.37), group 4 serum after 2 months' further injection with "old culture" vaccine *plus* 2 months' with "young culture" vaccine (14.2.38), groups 5 and 6 serum after 3 months' injection with double concentration "young culture" vaccine (12.5.38), filtered and unfiltered respectively, and the final group (7) serum following 2 injections of monovalent alum toxoid (27.6.38). Each group was subdivided into 3 sub groups of 10 mice for injection with 1, 2 and 3 A.L.D. of 24 m a living culture respectively (1 A.L.D. = 35 million). The suspensions were prepared from 5 hour agar slope cultures and viable counts made from broth dilutions.

Against 1 A.L.D. all sera gave complete protection throughout the 14 day test period except to 2 animals in group 5. With 2 A.L.D. all the control mice which received no serum died in less than 10 hours and no significant protection was afforded by the normal horse serum. On the other hand, 9 of 10 animals in group 3 survived for more than a week, while 8 were alive and apparently well when released after 14 days (mean = 288.5 ± 32.2 hours). While immunisation of the horse had been continued thereafter, though with an antigen prepared in a different way, the protective antibodies, including the α antitoxin, apparently decreased, since only 4 of the 10 animals in group 4 (mean = 192.2 ± 45.9 hours) and 2 of 10 in group 5 (mean = 109.4 ± 46.2 hours) survived throughout the 14 day test period, although only in group 5 was the decrease in protection statistically significant. Six of 10 animals in group 6, receiving the same serum as those in group 5 but without Seitz filtration, survived throughout (mean = 217.8 ± 47 hours). This might seem to indicate that the antibodies concerned with this protection were in a measure removed by Seitz filtration, although no decrease in α antitoxin titre could be detected, but the difference in protection is not statistically significant. The protective power of the high titre antitoxic serum (280 units) used in group 7 (mean = 267 ± 33.9 hours) was not striking or significantly greater than that of the unfiltered serum taken a month before (8 units) or than that of the serum used in group 3 (55 units).

When the dose of living culture was increased to 5 A.L.D., only two sera gave significant protection, that in group 3 (mean = 216.4 ± 41.1 hours) and that in group 7 (mean = 153.2 ± 33.2 hours). Both these sera had higher unitages of α antitoxin than those which were ineffective. That the α antitoxin unitage is a precise measure of the efficacy of the serum was not suggested, however, by this test, since 80 per cent of the animals receiving 280 units died in less than 8 days, while only 40 per cent of those receiving 55 units died in this time, and 50 per cent survived throughout the 14 day test period. This difference in protection is not statistically

significant, but indicates only that the serum with higher unitage of a antitoxin was not more effective than the one with lower unitage.

This experiment was carried out also with Wood 46, Blasko and Ryckman strains. With Wood 46 and Blasko, none of these sera was protective against more than 1 or 2 A.L.D. With Ryckman, the controls receiving 5 A.L.D. had a mean survival time of 5.10 ± 0.06 hours and significant protection was obtained with the serum of 6.10.37 (55 units, mean = 148.1 ± 48.7 hours) but not with that of 27.6.38 (280 units, mean = 26.2 ± 11.2 hours). For comparison, the results of these tests, in which the two sera effective against 5 A.L.D. of 24 m.a. were used, are summarised in fig. 4.

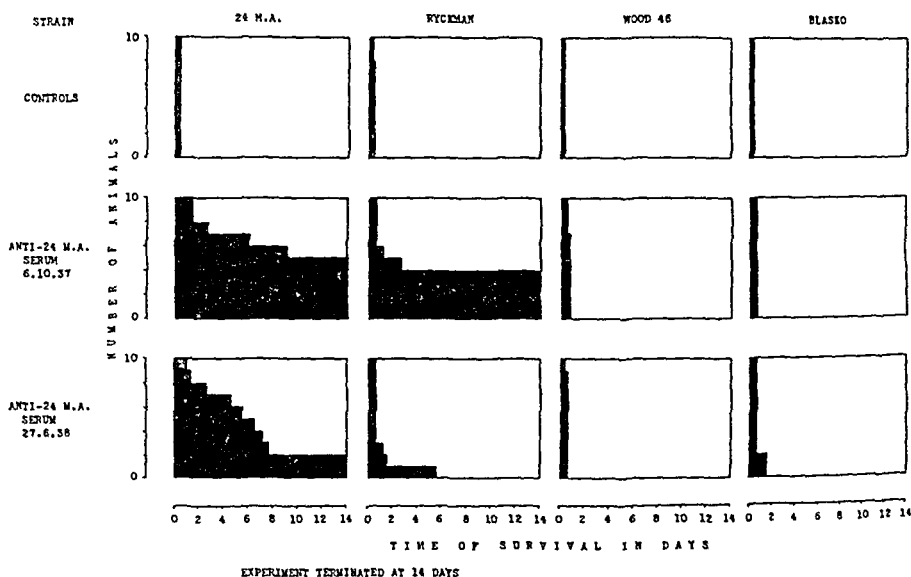


FIG. 4.—Protection with anti-24 m.a. sera against 5 A.L.D. (5-hour cultures).

The data show that anti-24 m.a. serum (6.10.37) gave better protection against the homologous culture than against any of the other 3 strains used, although the protection against Ryckman strain was significant.

As stated before, these tests were carried out with young (5-hour) agar slope cultures, and it seemed desirable to test overnight slope cultures also. Only the bleeding of 6.10.37 was used against 24 m.a. and Wood 46 strains for this test, which was carried out with each strain in the following way.

Forty mice were divided into three groups of 10 and two of 5. One group of 10 received 1 c.c. of antibacterial 24 m.a. serum (6.10.37). This group together with 10 controls, received 0.5 c.c. of the standard culture

suspension. Ten controls received 0.5 c.c. of the same suspension diluted 1/5, five 0.5 c.c. of suspension diluted 1/3, and five 0.5 c.c. of suspension diluted 1/8.

The results are shown in fig. 5.

The series of controls shows that 0.5 c.c. of undiluted suspension was equivalent to 5 A.L.D. of culture 24 m.a., and perhaps somewhat less of Wood 46. The controls receiving undiluted culture had a mean survival time of 4.21 ± 0.16 hours and 4.72 ± 0.17 hours respectively. Statistically significant protection was afforded by serum anti-24 m.a. (6/10/37) against this dose of both cultures but there can be no doubt that the protection was greater against the homologous strain than against the heterologous, and suspensions from 5-hour and 18-hour agar slope cultures seem fundamentally the same so far as these two strains are concerned.

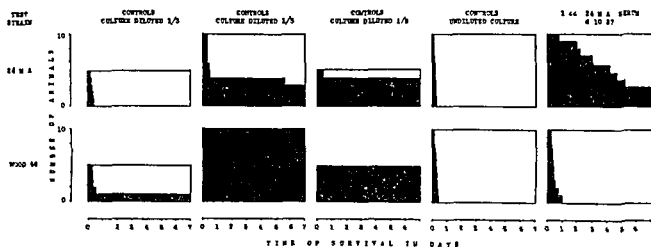


FIG. 5.—Protection with anti-24 m.a. serum against 5 A.L.D. (18-hour cultures)

DISCUSSION

Two salient points emerge from the data here presented. First, the α antitoxin titre is not completely correlated with the protective power of these horse sera against experimental infection in mice, secondly, there is a strong suggestion of specificity in the protection obtained with monovalent sera. The conclusion therefore appears warranted that the protection afforded by these sera is not due to their content of α antitoxin but to the presence of unidentified antibodies. The concentration of the antigens which stimulate formation of such antibodies may of course be parallel to that of the α toxin in the particular preparations employed for immunisation, but this need not be so. It seems likely, then, that the level of α antitoxin will be a good or poor index to the protective power of a serum according to the relative proportions of α toxin and of other antigens in the material used for immunisation and to the degree of response of different animals to the several antigens. Such an interpretation would afford sufficient explanation of the divergent experimental results reported in the literature from time to time.

Another point which is clearly shown by these experiments is that the dose of living culture used in lethal tests is of the utmost importance. Even normal horse serum, the low α antitoxin titre of which suggests the minimum experience by the horse of staphylococcus antigens, has been found repeatedly to protect against 1-2 A.L.D. On the other hand a massive dose may be overwhelming even to an animal with a high titre of antibodies. Consequently enough groups of control animals receiving different doses of culture should always be included to determine as closely as practicable the number of average lethal doses administered to the protected animals.

With regard to specificity of protection, it will be observed that the number of strains tested so far has been small, and that even in this group some crossing has been observed. Any number of types may of course exist, with unknown degrees of crossing. Moreover, in dealing with pyogenic micro-organisms of relatively low virulence, it is an open question whether 5 A.L.D. of one strain is in fact equivalent to 5 A.L.D. of another. In the absence of rules, some arbitrary standard must be set up.

For all these reasons there is need for caution in making generalisations from the data and particularly in attempting application to clinical infection which cannot be compared with the injection into mice of large doses of viable staphylococci. On the basis of these results, however, one is inclined to stress the use of polyvalent sera in large volume without recourse to rule-of-thumb considerations of thousands of units of α antitoxin.

SUMMARY

1. Sera prepared by injection of two horses with washed formalinised vaccines made from two toxigenic strains of staphylococci protected mice against experimental infection with approximately 5 average lethal doses of the homologous culture. Protection was not obtained with either serum against this dose of the heterologous strain.

2. One of these sera protected against 5 A.L.D. of one of 4 other heterologous strains tested; the other afforded some protection against another of these strains.

3. The α antitoxin titre was found to be a poor index of the protective capacity of these sera.

4. The protection obtained with these sera was independent of the antileucocidin titre as measured by the method of Valentine.

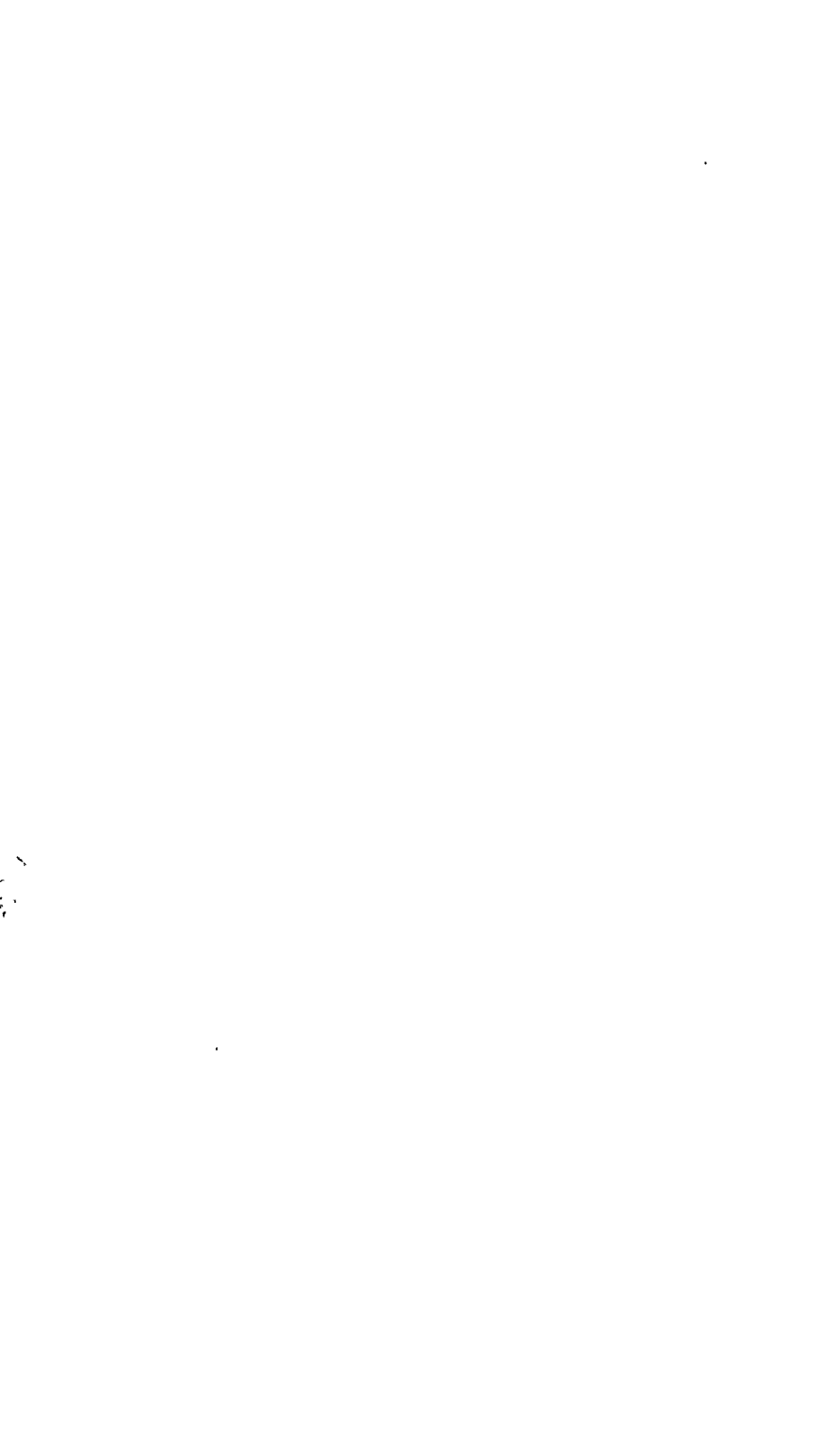
5. Absorption of agglutinins from one serum showed that the protection was not dependent upon these antibodies.

We wish to acknowledge with thanks our indebtedness to Dr Donald T. Fraser, Associate Director of the Connaught Laboratories, for kindly

criticism and advice throughout this investigation, to Dr F. C. O. Valentine, London Hospital Medical College, for titration of the antileucocidin content of sera, to Dr Mary A. Ross, Department of Epidemiology and Biometrics, University of Toronto, for direction in the statistical analysis of the data, and to Dr E. G. Kerslake, Veterinarian of the Connaught Laboratories, for immunisation of the horses.

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FAMILIAL CEREBELLAR HYPOPLASIA AND
DEGENERATION IN HEREFORD CALVESJ. R. M. INNES, DOROTHY S. RUSSELL *
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Baron Institute of Pathology, the London Hospital*

(PLATES XLIX-LII)

THE condition described in this paper appears to affect cattle of the Hereford breed only. This breed, which has probably been in existence for over 300 years, is remarkably pure. The Herd Book has been closed since 1884 and registration is now limited to the progeny of sires and dams entered at that time. The annual export of most of the outstanding sires of the herd to other countries has led to the retention in England of a relatively small number for breeding purposes. Further, the restriction of the choice of sires has resulted in a high degree of both inbreeding and line-breeding. Although attention is now drawn for the first time to the occurrence of familial cerebellar hypoplasia in English cattle, there is little doubt that it has been known to farmers for many years but has hitherto escaped investigation.

Source of material

Material from five pure-bred Hereford calves suffering from the condition has been examined histologically. They came from three different and widely separated farms. Calf 1 from the first farm was one of three, all females, which were born in 1936, showed similar symptoms and were the offspring of cows sired by the same bull, and part of a herd of fifteen animals. After disposal of the bull no further cases occurred nor did the condition occur in the progeny of other cattle outcrossed with Shorthorns. Our examination was confined to a few pieces preserved from the brain. Calves 2 and 3 were two of three calves (2 females and 1 male) affected at a second farm in 1939. On this farm two affected calves (both females) had been observed in 1938. One of the calves in 1938 and one in 1939 were the progeny of the same cow. A similar disease had been seen ten years earlier and had been eliminated by disposal of the bull. Calves 4 (male) and 5 (female) came from a third farm. The two cows concerned—in a herd of 20—had had normal progeny previously by the bull responsible for the diseased calves

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Information was also received concerning the occurrence of the disease on a fourth farm, although no cases were obtainable. The herd consisted of 25 cows of which 20 were pure-bred Herefords, and affected progeny again occurred only in the latter. Five affected calves were born in 1938 and six in 1939, all being females except one.

Clinical summary

The same symptoms were present in all the calves observed. The animals, when seen at birth and later, appeared stuporose and were unable to rise. They lay on their side with their limbs and neck stiffly extended (fig. 1), and if placed on their feet collapsed immediately, often backwards. Balance was maintained for a little longer if the animal was placed in the abnormal posture shown in fig. 2. Rigidity disappeared at times and then there were convulsive movements which seemed to be attempts to get up. In the more successful of these the calves sometimes adopted the same method of getting to their feet as a foal; that is, they raised themselves on their fore legs first. Occasionally they might get as far as the sitting position but soon collapsed again. There were no fits. Opisthotonos was sometimes conspicuous and periodically there were tremors of the head. Nystagmus was seen in one case only. The animals readily responded to the call of their dams by bawling and had no difficulty in sucking. In no instance has any clinical improvement been seen, although there was apparently no difficulty in keeping them alive by careful attention and bottle feeding. The calves were killed when from 1 to 20 days old.

PATHOLOGICAL EXAMINATION

The necropsies were made immediately after death and disclosed no abnormality apart from the brain. The material reserved for further examination was fixed and hardened in 4 per cent. saline-formaldehyde.

Macroscopic appearance of brain

Compared with a control specimen (fig. 3) the cerebellum was abnormally small (figs. 4 and 5), all the folia showing some degree of wasting. On section the texture was tough and leathery. The brain stem and cerebral hemispheres were normal, both superficially and on section.

Microscopic examination of brain

Methods. Blocks from different parts of the brain were embedded in paraffin and sections were stained with Ehrlich's hæmatoxylin and eosin, Weigert's iron hæmatoxylin and van Gieson's mixture, Weigert's fuchselin and neutral red for elastic fibres, phosphotungstic acid hæmatoxylin, toluidin blue for Nissl bodies, and Loyez's hæmatoxylin method for myelin. Frozen sections were stained with Scharlach R and hæmatoxylin, Spielmeyer's method for myelin, Del Río-Hortega's silver carbonate methods for neurofibrils and for glial fibres and Penfield's modification for microglia and oligodendroglia.

Pathological changes are confined to the cerebellum and are similar in all the cases examined. The folia everywhere are narrower than in normal controls (figs. 6 and 7). The degree of wasting

CEREBELLAR HYPOPLASIA IN CALVES



FIG. 1.—Calf 3 female 2 days old. Enlargement from ciné film showing the animal lying on its side with rigid extension of the limbs.



FIG. 2.—Same case as fig. 1, showing the only upright position which could be maintained and this for a short time only.

CEREBELLAR HYPOPLASIA IN CALVES

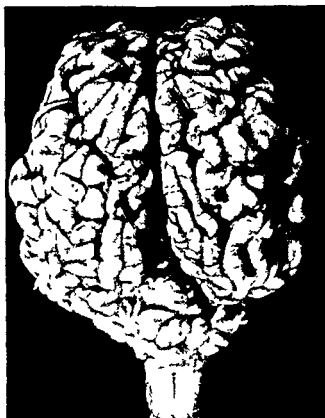


FIG. 3.—Brain of normal control calf 5 days old. Compare with figs 4 and 5.
 $\times \frac{1}{2}$ circa.

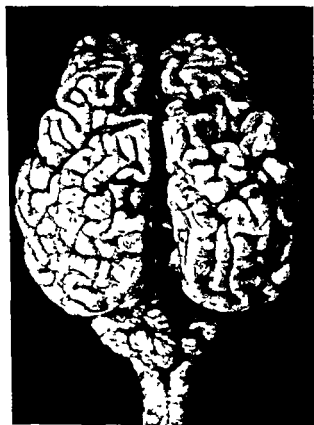


FIG. 4.—Brain of calf 2, female, 2 days old, showing small size of cerebellum.



FIG. 5.—Brain of calf 5, female, 8 days old.

varies slightly in different parts of a specimen and between the different cases. It is associated with gross disorganisation of the normal cortical structure (*cf.* figs. 6 and 7). There is no inflammatory or other change in the adjacent leptomeninges. Beneath the pia the foetal granular layer is still present in places but is seldom more than three cells deep. The molecular and granular layers are both abnormally thin. Their boundaries are somewhat blurred through the presence of an excess of cells in the molecular layer and the almost complete absence of Purkinje cells. The cells in the molecular layer are principally round or oval and are about twice the size of the granule cells (fig. 8). They are fairly evenly distributed throughout the layer. Each contains a relatively large, eccentric nucleus which, in silver preparations, has pale grey nucleoplasm and a single large central nucleolus. About the nucleus is a narrow, uneven rim of argentophil cytoplasm which occasionally extends to form from one to three delicate unbranched processes. Rarely these cells are bipolar; they are then orientated in either the vertical or horizontal direction. From their appearance they are undoubtedly neuroblasts, but they show little evidence that they are precursors of the granule cells proper and they are considerably larger than the cells of either the foetal granular or inner granular layer. Apart from these neuroblasts the molecular layer contains occasional microglial cells of normal appearance and astrocytes whose processes contribute to a dense vertical gliosis (fig. 9). The greater part of these glial fibres are, however, derived from Bergmann cells occupying the upper boundary of the granular layer. Amongst them an occasional Purkinje cell may be found. These are usually shrunken and deformed, their dendrites being few, coarse and angular. In Nissl preparations they often show great pallor, loss of Nissl substance and karyolysis. In one instance a retraction ball was found upon the axon. Rarely neurones of the Purkinje type occupy the molecular layer or the depths of the granular layer. Most often the larger neurones in the granular layer are, as normally, of the Golgi type (fig. 8). These often show eccentricity of their nuclei and margination of their Nissl substance. A few neuroblasts, similar to those in the molecular layer, are also present in the granular layer. The granule cells proper are fewer than in normal controls; their nuclei are sometimes shrunken and pyknotic. In this layer also there is an excess of neuroglial fibres.

The white matter is somewhat reduced in amount. Both axis cylinders and myelin sheaths are well preserved except for occasional ballooning and droplet formation in the latter. The tangential fibres of the cortex are also preserved (fig. 10).

The central nuclei of the cerebellum appear normal. Comparison with sections from a normal calf 5-7 days old showed no differences

in the structure of the pons and medulla oblongata. In particular the inferior olives were similar in both.

No abnormality was observed in the spinal cord.

DISCUSSION

The changes in the cerebellum in these animals might be attributed either to an arrest of development, or to a degenerative process or to both. In support of the former theory is the relative paucity of cells in the granular layer, the excess of neuroblasts in the molecular layer, the absence of any but trivial destruction either of axis cylinders or of myelin and the normal appearance of the microglial cells. In favour of a degenerative process is the pronounced gliosis of the cortex and the evidence of degeneration in the surviving Purkinje cells and other neurones. Post-mortem degeneration can be absolutely excluded since the material was fixed within 20-30 minutes of the death of the animal. It is generally agreed, following Ramón-y-Cajal, that the granule cells are derived from the foetal granular layer of Obersteiner by the inward migration of these cells. The foetal granular layer in the diseased calves is no greater than in the controls and yet the inner granular layer is not of normal density. This might be attributed equally to destruction of granule cells or to defective formation (hypoplasia) of the foetal granular layer. The neuroblasts in the molecular layer are about twice the size of the granule cells and for the most part lack the bipolar character of these cells during migration. It is unlikely, therefore, that they are intermediate forms. They might be regarded as precursors of the Purkinje cells which are conspicuous by their absence from their normal position. The origin of the Purkinje cells has never been finally settled, though it is usually supposed that they have an independent origin from the inner mantle layer. While the occasional presence of recognisable Purkinje cells in the molecular layer is a normal feature in various animals (Ramón-y-Cajal, 1911) and is therefore without special significance in these cases, the histology of the molecular layer suggests that the origin of the Purkinje cells from the external granular layer is at least a possibility.

It might be argued, however, that the Purkinje cells had already reached their proper destination and subsequently disappeared through degeneration. The evidence of degeneration in the few existing Purkinje cells supports this. On the other hand, when many Purkinje cells have been destroyed it is usually possible to demonstrate the places which they occupied by the empty baskets of climbing fibres which persist at such sites, while destruction of their axones leads, in sections stained for myelin fibres, to the appearance of zones of pallor about the dentate nuclei. Neither

CEREBELLAR HYPOPLASIA IN CALVES

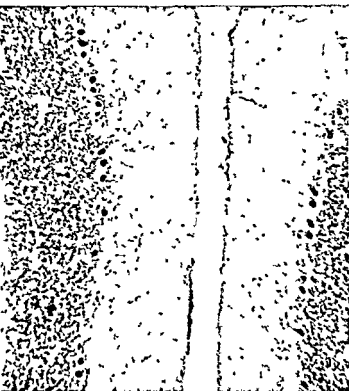


FIG. 6—Cerebellar cortex of normal control calf
Hæmatoxylin and eosin $\times 56$



FIG. 7—Calf 3 Cerebellar folium showing
atrophy, poor cortical pattern and absence
of Purkinje cells Hæmatoxylin and eosin
 $\times 56$

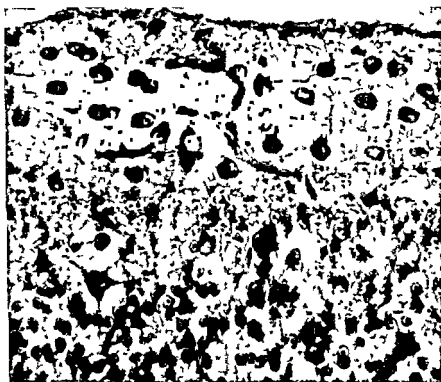


FIG. 8—Calf 3 Cerebellar cortex showing large neuroblasts in molecular layer
and a few in granular layer Golgi cell to left of granular layer Silver
carbonate $\times 330$

CEREBELLAR HYPOPLASIA IN CALVES

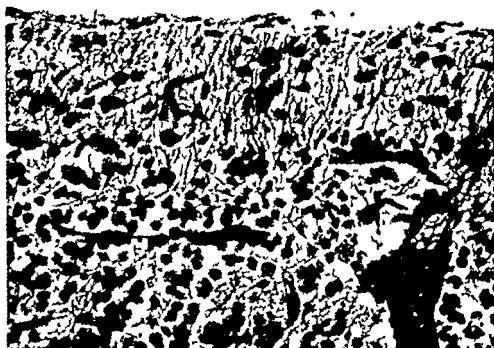


FIG. 9.—Calf 3. Cerebellar cortex showing gliosis of molecular layer. Silver carbonate $\times 345$.



FIG. 10.—Calf 3. Cerebellar cortex showing preservation of tangential fibres. Two portions of a degenerating Purkinje cell present to left of centre. Silver carbonate $\times 31$.

of these features was seen in the present cases. It seems probable, therefore, that there is deficient formation of Purkinje cells as well as degeneration of those that have been formed. The large neuroblasts in the molecular layer may represent immature Purkinje cells which have been delayed in transit. The possibility that they are morphologically abnormal precursors of the granule cells cannot of course be excluded. There appears sufficient reason to conclude that the pathological changes in the cerebellum in these calves is the result of arrested development in addition to degeneration of the cortex.

A similar condition has been recognised in kittens (Herringham and Andrewes, 1888; Brouwer, 1934). In both of these reports several kittens of the same litter were affected. While the histological condition described in the earlier paper appears to have been identical with that now described, in Brouwer's cases there was, in addition, an atrophy of the inferior olives and of the oral parts of the *nuclei pontis*. The clinical features in both batches of kittens were similar to those in the calves but of less severity. Congenital ataxia in a cat six months old was described by Langelaan (1907). The other five of the litter were unaffected. This case resembled the calves in respect of the cerebellar changes and the immunity of the inferior olives but differed from them in that there was degeneration of the spinocerebellar tracts. The case of cerebellar atrophy described by Robin (1911) in a calf of unspecified age and breed resembles our cases clinically, but differs pathologically in that the cerebellar changes appear to have been confined to the lateral lobes. In either of these there was advanced destruction of the folia in a basin-like depression. The microscopical appearances are not, however, described. One of a litter of congenitally ataxic puppies was reported by Risien Russell (1895). It is of interest that the parents were described as being healthy prize dogs. Histological examination in one puppy showed a condition of the cerebellar cortex which was probably identical with that in the calves except that the absence of Purkinje cells was not so complete.

An analogous condition almost certainly finds a place in human pathology. Congenital cerebellar ataxia was distinguished clinically from Friedreich's ataxia by Batten (1905) and has more recently been reviewed by Hallervorden (1937). While familial examples have been recorded, the onset of symptoms in such cases has usually been in the middle decades (Thorpe, 1935). Pathologically there is a primary degeneration of the Purkinje cells with their axones and a reduction, often uneven, of the molecular and granular layers (Holmes, 1907-08). Of greater interest is the report by Fraser (1880) of a familial cerebellar ataxia affecting a brother and sister in the second or third year of life and persisting to adult

life. Pathological examination of the male showed a diffuse cerebellar atrophy with great reduction of the cortex and loss, through degeneration, of a large number of Purkinje cells. In these familial human cases, therefore, the changes in the cerebellum appear to be purely degenerative and the aplastic features seen in the young domestic animals are lacking. Nevertheless, it is possible that the underlying cause is the same in both and that the observed differences are attributable simply to the onset of degeneration in the young animals at a time when development is incomplete. Scherer (1933) has reported three cases of non-familial cerebellar cortical atrophy in young infants. His first differs from the calves in that the cerebrum also was small and there was demyelination in the neo-cerebellum. The cortical changes in the cerebellum also were patchy. His second and third cases were histologically similar to the calves but bore a closer resemblance to Brouwer's kittens in that the inferior olives and the pons were also atrophied. In this they agreed with the case reported by Holmes.

The ætiology of human familial cerebellar ataxia is unknown; it would therefore be highly important if it were established that the condition in calves is due to a hereditary factor. The evidence supporting this possibility is highly suggestive.

Breeders of pedigree stock usually raise only those that are perfectly sound and in consequence when abnormalities appear the animals are discarded with as little comment as possible. Their attempts to fix certain types or characters, however, have occasionally produced structural defects in cattle, the most noteworthy being the so-called "bull-dog" calf, mainly of the Dexter breed. The available facts which support the idea that the disease is hereditary are :—

1. Defective calves were born when the Hereford bull was bred to Hereford cows but did not occur in Shorthorn crosses.

2. From this and because only a relatively small number of diseased cattle were born in any one herd the character might appear to be recessive.

3. More females were affected than males. This accords with the common finding that many defective inherited characters have a relationship to sex (Sewall Wright, 1934).

4. In two instances, replacement of a bull which appeared to be siring defective progeny was followed by cessation of the disease.

Further discussion is beyond the scope of this paper, but it is apparent that the subject merits attention from the genetical aspect.

SUMMARY

The clinical and histological features of five examples of familial cerebellar hypoplasia, arrested development and degeneration in calves are described. The pathological changes are confined to the cerebellar cortex.

In cattle the condition appears to be restricted to the Hereford breed and is possibly due to a hereditary factor which has emerged as the result of intensive inbreeding.

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THE FIRST EFFECTS ON MOUSE SKIN OF SOME
POLYCYCLIC HYDROCARBONS

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From the Imperial Cancer Research Fund, London

(PLATES LIII AND LIV)

CERTAIN of the polycyclic hydrocarbons are known to pathology through their capacity to cause malignant growths (Cook *et al.*, 1932; Cook, 1932; Barry *et al.*, 1935), to inhibit the growth of transplantable tumours (Haddow, 1935; Haddow and Robinson, 1937) and to behave as protoplasmic poisons (Polson, 1936; Mottram and Doniach, 1937, 1938). Their exact pathological action on cells within the living animal is still unknown. Many attempts have been made to describe microscopic changes after treatment with tar. Owing to its content of non-specific irritants none of these changes can be ascribed with certainty to a tumour-inducing hydrocarbon in tar. After the pure carcinogenic chemicals had been synthesised by Cook, Hieger, Kennaway and Mayneord (1932), it became possible to study the response of tissues to these substances free from the confusion due to solvents or impurities. Most previous observations, based on reactions to tar or pure compounds, have been concerned with the effect of repeated applications during the period which precedes the appearance of tumours. These studies have shown that repeated applications are followed by epithelial hyperplasia and hypertrophy of skin. Neither change is specific. Both are common to many chronic and healing lesions from different causes. A reaction that is specific in any respect has not been described. Yet it is probable that carcinogenic chemicals, like other chemical and physical agents, injure skin in a way that is peculiar to themselves. The response to a first treatment has been almost entirely neglected, though Orr (1938) noticed squamous hyperplasia one week after a single application of methylcholanthrene, and Page (1938) observed and measured the increase in size of nuclei and nucleoli soon after the application of methylcholanthrene and cholanthrene dissolved in benzene. An examination of this first step is essential to an understanding of the whole pathological action. Once the tissue has responded, it will not be the same

were tested to find one that is itself not injurious. In agreement with Orr I found that cold acetone does not affect the microscopic structure of mouse skin. Zenker-fixed paraffin sections were prepared except where stated; other fixatives were used for special purposes. Most of the staining was done with Ehrlich's hæmatoxylin and eosin and Weigert's iron hæmatoxylin and van Gieson or the ponceau S. modification. These combinations of fixation and staining were found to give by far the most clean-cut pictures. Bouin also was a suitable fixative and was used to show up certain granules. Both Bouin and sublimate-formol mixtures make the collagen of mouse skin swell and thus, by leaving a clear channel in a rather homogeneous matrix, dilated lymphatics are clearly revealed. Formol-saline is almost valueless for this material except after long fixation, and even then it fails to give good fixation of inflammatory changes in connective tissue.

The standard method used to examine a chemical was to clip the hair by hand, with curved scissors, from the nape of the neck of young mice. Any mice cut by accident or found to be badly infested with lice were discarded at this stage. A pair of binocular loupe spectacles is a great help in making this selection. One drop of a 0.3 per cent. solution or three drops of a 0.1 per cent. solution in acetone was applied, time being allowed in the latter case for the acetone to dry off between the drops. Several drops of the dilute solution had to be used when the chemical was not soluble up to 0.3 per cent., the dilution which it is customary to employ in most experiments on carcinogenesis. No difference was found when these two ways of applying the chemicals were compared. The solution was applied to the skin by means of a glass pipette with a double bend and drawn to a capillary point. In these pipettes one can hold the acetone under control. Solutions were applied either as drops or by touching the skin with the point of the pipette and allowing the fluid to flow as far as needed. In this way it will spread more evenly. About 1 c.c. of a 0.3 per cent. or 3 c.c. of a 0.1 per cent. solution was required for 32 mice and no further application was made. Four or three mice per day were killed on the four days following treatment, and after that on alternate days up to the 10th or 12th. The treated skin with some normal skin around it was cut out and, without stretching, was pinned down with hedgehog quills on firm thin pieces of cork. The tissue was then fixed in Zenker's fluid for 24 hours. After fixation a strip including treated and untreated areas was cut out for sections, which were prepared according to the usual paraffin embedding technique. Each strip was cast along the edge of an L-shaped metal mould and was cut at right angles to the skin surface. At this stage and in the final preparations, if very well dried, the treated area could be identified by its clipped hair and by the longer hair on either side of it.

Group A. Highly active carcinogens with short latent periods

Methylcholanthrene (3 consecutive drops of a 0.1 per cent. solution in acetone)

24 hours after application (fig. 2). Slight swelling of the epidermis up to twice normal, due to increase in size of individual cells, and increase in the diameter of the nuclei up to 1.4 times normal, become visible. Chromatin tends to collect in the nucleoli and nuclear membrane. A few nuclei are visible in unfixed frozen sections. The cytoplasm is swollen and vacuoles begin to appear on one side of or around the nuclei. The whole cell stains poorly.

Few mitotic figures are to be found but there are numerous paired nuclei. Signs of differentiation are absent. There is slight hyperæmia of vessels superficial to the panniculus carnosus and a slight increase in the number of polymorphonuclear leucocytes, macrophages and mononuclear and unidentified cells.

2 days after application (fig. 3). Many cells are now three times, and nuclei 2-3 times, their normal diameter. Vacuolation of cytoplasm and nuclear distortion especially near the surface are noticeable. The majority of nuclei are visible in unfixed frozen sections. Multiplication of cells has taken place, leaving the epidermis 2-4 times thicker and 4-5 cells deep. This increase in the number of cells appears to be due to direct division, since on the previous day paired nuclei were far more numerous than cells in mitosis. Mitotic figures are now present but are not numerous. Variation in size of cells and nuclei begins to attract attention. Signs of differentiation are not often found. The surface is covered by a deep dense layer of keratin which is strongly eosinophile. The rest of the epithelium stains feebly. Acute inflammation in the sub-epidermal connective tissue has increased. Capillary vessels are swollen and engorged with blood. In macroscopic specimens hyperæmic vessels make a ring around the treated area on the reflected undersurface of the skin and subcutaneous tissue. Polymorphonuclear leucocytes are abundant; mononuclear cells, fibroblasts and unidentified cells have increased in number. Collagen fibres are swollen and appear to be fragmented and more widely separated than normal. Lymphatic capillaries are dilated, indicating a state of cedema (Pullinger and Florey, 1935). They can be identified by valves which often appear in sections. The lymphatics lie along the side of the hair follicle which makes an acute angle with the skin surface.

3 days after application (fig. 4). The contrast between the two preceding phases and that seen on the 3rd day is very striking. It is partly dependent upon differences in colour due to avidity for stains but other essential points can be seen in the drawings. Many cells, nuclei and nucleoli have enlarged still more, the cells and nuclei to 5 or 6 times normal, and great variation in size is apparent. The majority of the nuclei are visible in unfixed frozen sections. The epidermis is up to 15 times thicker and 4 or 5 cells deep. Occasionally the nuclear membrane is wrinkled or distorted. Chromatin now stains deeply. A small number of dead cells with pyknotic nuclei and hyaline cytoplasm are found and a number of nuclei have undergone karyolysis but more appear to have recovered to some extent from the action of the hydrocarbons. They are nevertheless distinctly abnormal. Mitoses are numerous. The cytoplasm stains deeply, partly with hæmatoxylin, partly with eosin. It is sometimes vacuolated and there is often a well defined

EFFECT OF POLYCYCLIC HYDROCARBONS ON SKIN

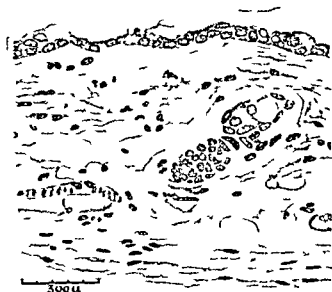


FIG 1 —Normal skin of an eight weeks old mouse



FIG 2 — Mouse skin 24 hours after the application of three drops of a 0.1 per cent solution of methylcholanthrene in acetone

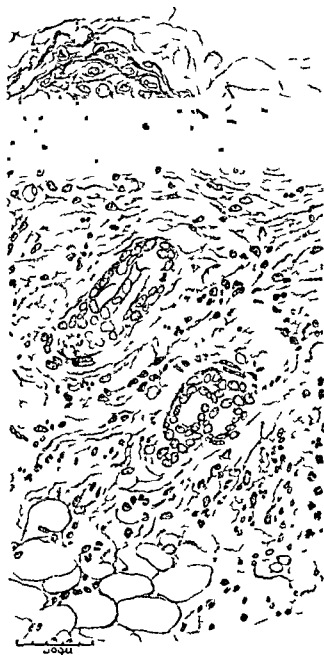


FIG 3 — Mouse skin two days after the application of three drops of a 0.1 per cent solution of methylcholanthrene in acetone

The magnification of all figures is the same

edge to the vacuole, stained a little deeper than the rest of the cytoplasm, which is diffusely and finely granular. Signs of differentiation have appeared in the form of intercellular bridges and keratohyalin granules. Cells near the surface are flattened and keratin has increased so much that it often blocks the mouths of hair follicles so that they and the sebaceous glands begin to undergo pressure atrophy. Inflammation in connective tissue is not increased beyond that seen on the previous day. The fine diffuse granularity makes one expect to find cloudy swelling by examining unfixed frozen sections. This expectation, however, is not confirmed. There is no "ground glass" opacity and no appreciable clearing of the cells by adding dilute acetic acid. In addition to the fine diffuse granularity, minute discrete localised granules appear on the 3rd day and still more on the 4th day. They are visible chiefly to one side of and around the nucleus in unfixed frozen sections and in material fixed in Bouin's and Zenker's fluids, but not in formol-saline, sublimate-formol, Champy's fluid or alcohol. They stain faintly with picric acid and eosin and brilliant red with Mann's eosin-methyl-blue stain. They fail to retain Gram's stain and it is thus improbable that they represent hyaline droplet degeneration. They do not stain with carmine, osmic acid or Heidenhain's iron-haematoxylin. As the cells containing them proceed to the surface, variation in the size of the droplets occurs and some become very much larger. They are visible in the stratum granulosum together with the basophile keratohyalin granules. A further cytoplasmic degeneration is also displayed, but more often on the 4th day. Clumps of rounded or oval eosin-staining cytoplasm, sometimes granular, sometimes hyaline, separate out in some of the cells (fig. 5).

4 days after application (fig. 5). The majority of the cells and nuclei are smaller, the largest 4 and 3 times normal respectively; the epidermis is reduced in thickness from 15 to 10 times normal. Nuclei and cytoplasm stain more deeply. Some of the very large cells have a tendency, even when fairly deeply situated, to be arranged with the longest axis parallel to the surface. Vacuolation is less conspicuous. Signs of differentiation and the natural progress towards cell death have increased. Follicle mouths are freer of keratin but sebaceous glands have not yet begun to regenerate. Cell division is active. Eosinophile-granule degeneration and clumped cytoplasm are still to be found (see the two big cells in fig. 5). There is a reduction of the hyperaemia, oedema and cellular exudate in the connective tissue.

6th and 10th days after application. From the 4th day onwards a gradual reduction in the number and size of cells and nuclei takes place. No very large cells are seen later than the 6th day and the variation in size, so obvious before, disappears.

The large cells gradually reach the surface and are shed after natural degeneration. Other abnormalities include an increase in keratin and keratohyalin. Return to normal proceeds from the centre of the treated patch and is usually less advanced at the edges. Hair follicles reopen and sebaceous glands begin to appear again as keratin is produced in less amount and the excess is cleared from the surface. By the 10th day there is a return to normal in patches. Signs of inflammation gradually decrease.

Benzpyrene, 9:10-dimethyl-1:2-benzanthracene, 3:4:5:6-dibenzcarbazole, 2-methyl-3:4-benzphenanthrene

This description of the first reaction to methylcholanthrene applies also to the same dose of a sample of benzpyrene which gave rise to epithelial tumours in 100 per cent. of mice; it applies to the same dose of 9:10-dimethyl-1:2-benzanthracene with a slight variation in rate of progress of the lesion, which developed more rapidly in some and more slowly in other mice; a greater number of dead cells can be found. The same dose of 3:4:5:6-dibenzcarbazole causes the same response in a few of the mice as is seen on the 2nd and 3rd days after methylcholanthrene; a larger dose—45 consecutive drops—was followed by the characteristic reaction in all the mice; no intermediate dose was tested. A dose of 2-methyl-3:4-benzphenanthrene fifty times larger was required to reveal the same response as 3 drops of a 0.1 per cent. solution of methylcholanthrene, benzpyrene or 9:10-dimethyl-1:2-benzanthracene; it appeared in the majority of mice on the 5th and 6th days; inflammation in the connective tissue was less intense.

Group B. Less active carcinogens

The response to these chemicals was examined in the same way as before. None gives rise to the characteristic 2nd and 3rd day lesions. 1:2:5:6-Dibenzanthracene, 1:2:5:6-benzfluorene, 3:4-benzphenanthrene and 3:4:5:6-dibenzacridine produce swelling of cells and nuclei, vacuolation of cytoplasm and poor staining capacity. Increase in cell size and variation in size persist to the 4th day at least, but are never so great as those due to the action of members of group A. Multiplication of cells occurs slowly. By the 4th day the epithelium has increased in thickness to 4 cells deep and has differentiated, except after 3:4-benzphenanthrene, when no cell multiplication or differentiation follows. All these changes develop to the greatest degree in response to 1:2:5:6-dibenzanthracene. Persistence of cell multiplication and differentiation until the 4th day serves to distinguish the chemicals which cause it from members of the next group of non-carcinogenic hydrocarbons.

EFFECT OF POLYCYCLIC HYDROCARBONS ON SKIN



FIG. 4.—Mouse skin three days after the application of three drops of a 0.1 per cent. solution of methylcholanthrene in acetone.



FIG. 5.—Mouse skin four days after the application of three drops of a 0.1 per cent. solution of methylcholanthrene in acetone. Two large cells contain clumped hyaline cytoplasm and minute discrete eosinophile granules.

3-Methyl-1 : 2-benzanthracene, 7-methyl-1 : 2-benzanthracene, chrysene and 1 : 2 : 5 : 6-dibenzacridine cause slight swelling and vacuolation in the first 24 hours only. The swelling does not increase or even persist. Cells shrink again, become more uniform in size and by the 4th day the skin is normal or shows only a little more keratohyalin than usual. This group is being examined further to ascertain the effect of repeated treatment at intervals of a few days. It has been noted already that degeneration and swelling of the degree that is characteristic of group A occur in the course of ten days after three separate applications at 3-4-day intervals of 2-methyl-3 : 4-benzphenanthrene.

Group C. Non-carcinogenic hydrocarbons

Phenanthrene, 1 : 2-benzanthracene, pyrene and fluorene fail to give reactions resembling those due to active carcinogenic hydrocarbons when an equal dose or fifty times this dose in acetone is used. All cause epithelial damage shown by slight swelling and vacuolation of cells followed by slight hyperplasia and return to normal by the 4th day.

Group D. Non-carcinogenic derivatives

Treated with equal doses of 2 : 6-dimethyl-1 : 2-benzanthracene, 10-isopropyl-1 : 2-benzanthracene, 3 : 7-dimethyl-1 : 2-benzanthracene, 1 : 2 : 3 : 4-dibenzanthracene, 1 : 9-dimethylphenanthrene or 4-hydroxy-3 : 4-benzpyrene, mouse skin fails to give the reaction characteristic of the carcinogenic hydrocarbons.

Group E. Non-carcinogenic compounds

Naphthacene produces no change in the epidermis when 10 drops of a 0.037 per cent. (saturated) solution in acetone is used. Triphenylene and 4-methyldiphenyl cause slight swelling, vacuolation and hyperplasia of the epidermis. The change does not resemble that due to the active carcinogenic compounds.

Group F. Solvents and irritants

Olive oil, mouse fat, liquid medicinal paraffin

A large dose was tested by applying drops at half-hourly intervals for a period of three hours. Olive oil and liquid mouse fat (warmed to 36° C.) give rise to inflammation of the subepithelial connective tissue, vacuolation of epidermal cells, hyperplasia and parakeratosis. Liquid medicinal paraffin causes lesions similar in kind but less in degree. Apart from cell proliferation and inflammation there is no resemblance between these changes and those caused by the active carcinogenic hydrocarbons.

Chloroform, benzene, ether

Three consecutive drops, equal to about 0.1 c.c. delivered from a capillary pipette, of chloroform or benzene cause severe damage followed by healing. Chloroform actually kills the surface epithelial cells; benzene causes swelling of nuclei and "ballooning" of the cytoplasm. Subepithelial connective tissue is inflamed. Healing is accompanied by hyperplasia and hyperkeratosis. Six drops of ether, equal to about 0.2 c.c., are required to damage the epithelial cells. The damage is shown by slight swelling followed by slight thickening and hyperkeratosis. The changes do not resemble those due to active carcinogenic compounds.

Cantharidin, monochloracetone

Various dilutions of these two substances were tested as examples of skin irritants. Both destroy epithelium, cantharidin in a dilution of 0.01 per cent., monochloracetone when undiluted. Connective tissue is inflamed. Healing is by regeneration from basal cells at the edge of the injury.

Summary

1. The first response to treatment of mouse skin with some carcinogenic and non-carcinogenic polycyclic hydrocarbons is described.

2. One application of methylcholanthrene, benzpyrene, or 9:10-dimethyl-1:2-benzanthracene is injurious and is followed on the 2nd, 3rd and 4th days by a characteristic and possibly specific reaction. Squamous epithelial cells and nuclei swell, the cytoplasm becomes vacuolated and multiplication at first by direct and later by indirect division follows. The increase in size of cells and nuclei is progressive, further cytoplasmic degenerations appear in some of the cells while others show signs of recovery. Cloudy swelling is not detectable and premature death of cells is relatively rare.

3. A similar reaction follows the application of a large amount of 3:4:5:6-dibenzcarbazole on the 2nd, 3rd and 4th days and, in the majority of mice, on the 5th and 6th days following like treatment with 2-methyl-3:4-benzphenanthrene.

4. Treatment with small and large amounts of non-carcinogenic hydrocarbons and other chemicals shows that all those tested except naphthacene are injurious to the squamous epithelium of the mouse. None evokes the reaction which follows the application of the active carcinogens.

It is a pleasure to acknowledge the generous help of Professors E. L. Kennaway and J. W. Cook, who chose and provided most of the hydrocarbons tested. Methylcholanthrene, 3:4-benzpyrene and 1:2:5:6-dibenzanthracene were obtained from L. Light & Co. and phenanthrene from British Drug Houses.

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POST-MORTEM GLYCOLYSIS

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GLYCOLYSIS in blood is brought about by an intracellular enzyme and its rate is accelerated by the addition of alkali as shown by Claud Bernard (1877) or by raising the pH by removal of CO_2 as shown by Lovatt Evans (1922). Tolstoi (1924) found that a decrease in temperature lowers the rate of glycolysis *in vitro* and that during the first 24 hours it is not affected by bacterial contamination. Maclean and Weir (1915) showed that the presence of large numbers of leucocytes in the blood accelerates the rate of glycolysis, and Macleod (1913) demonstrated the retarding effect of oxalate on the process. The glycolytic power of blood *in vitro* has been investigated with contradictory results. Maclean (1915) and Tolstoi (1924) showed that normal and diabetic bloods undergo glycolysis equally well. Maclean's figures for the rate of disappearance of sugar from normal blood *in vitro* was 16.3 and for diabetic blood 18.29 mg. per 100 ml. per hour. He did not, however, take into account the deceleration of the reaction with the alteration of concentration of the reacting compounds. Denis and Giles (1923) reported a diminution in the glycolytic power of diabetic blood, and Lépine (1909) found that glycolysis was absent in the blood of depancreatised animals but was restored by the addition of pancreatic extracts.

The present investigation was undertaken to find out the possibility of determining, by post-mortem blood sugar estimations, whether a subject had died from diabetic coma or coma due to insulin overdosage. In the former condition hyperglycæmia would be present at death, in the latter hypoglycæmia. It was hoped that a definite rate of glycolysis might be found in the sugar remaining in the blood of the body after death, and that the variations in this rate, caused by other factors such as decrease in temperature due to the body cooling, could be determined.

The complex changes taking place in the body after death make a direct study of the rate of glycolysis within the cadaver impracticable, and we have therefore confined ourselves to estimations of the changes of the sugar content of the blood and cerebrospinal fluid after death and indicating how these may have arisen.

METHODS

Samples

Blood. Where possible, part of the sternum was removed and samples of blood taken from the right auricle, pulmonary artery and aorta. In other cases a sample of blood was taken from the pulmonary artery with a Pasteur pipette through the second intercostal space on the left side. Potassium oxalate was used as anticoagulant.

Cerebrospinal fluid. Samples were always obtained by cisternal puncture.

Liver. To determine the amount of glucose in liver a specimen was taken from near the entry of the hepatic veins into the vena cava and roughly dried on blotting paper. One g. of this liver was ground up with 1 ml. of distilled water. The sugar estimations were done on the resulting supernatant fluid.

Estimations

Sugar (total reducing substances). Sugar was estimated by the method of Folin and Wu, the protein being precipitated as soon as possible after collection. Where the values to be estimated were over 500 mg. per 100 ml., the specimen was diluted 10 times.

True (fermentable) sugar. Absolute estimations of sugar were made by fermenting with relatively large quantities of yeast as recommended by Hiller, Linder and Van Slyke (1925), and then determining the non-glucose-reducing substances by the method of Folin and Wu. A standard solution of 200 mg. of glucose per 100 ml. was used as a control to ensure the activity of the yeast preparation.

Non-protein nitrogen. The micro-Kjeldahl method was used for the determination of non-protein nitrogen.

RESULTS

Fifty cases were examined in all, including eight diabetics, seven of whom died in coma. Two cases of fractured skull with raised intracranial pressure and hyperglycaemia are included amongst the non-diabetics.

The results are discussed in the following groups:—(a) non-diabetic subjects; (b) non-diabetic subjects with hyperglycaemia developing in the right side of the heart after death; (c) diabetic subjects; (d) some experiments on glycolysis *in vitro*.

Results in 34 non-diabetic subjects

Glycolysis proceeds very rapidly in non-diabetic subjects in both the blood and the cerebrospinal fluid. In twenty-four cases maximum glycolysis had occurred when the first estimation was made (7-16 hours after death). In eight cases where we had immediate access to the body we have been able to show that the process is complete within $3\frac{1}{2}$ -7 hours after death. This is made clear in table I, where these results are shown. The post-mortem increase in non-glucose reducing substances (shown in table III) accounts for the fact that the figures do not fall below 20-30 mg. per cent. The internal body temperature was recorded on a

thermometer plunged into the liver. Cooling took place at room temperature. Refrigeration makes little difference to the rapidity of glycolysis as the process is almost complete before the internal organs are cooled. Cases 4 and 8 show a slight rise in the blood sugar values 24 hours after death but, as will be shown later, this is due to an increase in the non glucose reducing substances resulting from autolysis.

TABLE I

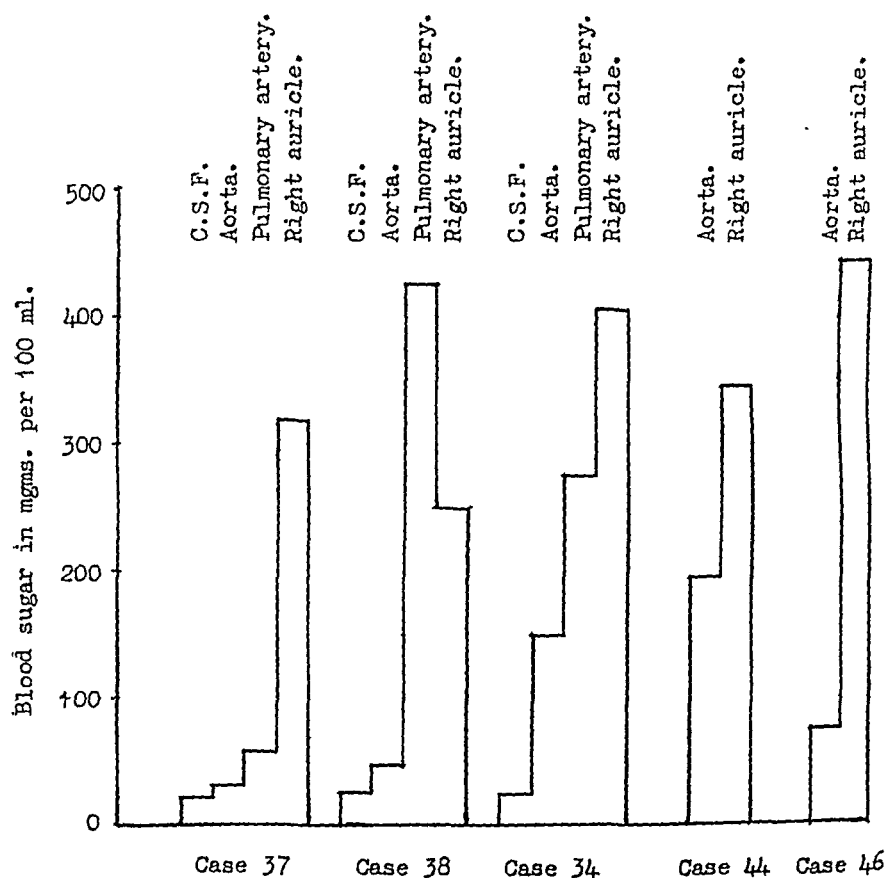
Post mortem glycolysis in non diabetic subjects

Case no	Time after death in hours	Temperature in liver (degrees Centigrade)	Blood sugar in mg per 100 ml	Cerebrospinal fluid sugar in mg per 100 ml
2	7 11½	35.0 33.0	27 27	
4	12 hours before death 2½ 4½ 6½ 24	blood sugar 122 mg per 100 ml 38.0 36.0 32.0 16.0	116 25 36 53	26
5	2 4½ 6		60 54 25	
6	4 23 28	40.0 24.0 22.5	25 35 27	
8	3½ 6½ 29	34.5 31.0 17.0	32 30 36	38 31 33
10	4 6½	34.0 32.5	23 30	
13	2½ 4 6½ 23½	34.0 30.0 27.0 16.0	55	71 55 53 50
27	8 27		26 23	25 21
28	16	28.0	25	17
29	16		46	21

Two cases of hyperglycæmia following fracture of the skull and cerebral hæmorrhage were studied. Glycolysis did occur but was slower. In case 21, 5½ hours after death, the blood sugar in the left ventricle was 167, in the right ventricle 147 and in the cerebrospinal fluid 88 mg per 100 ml. Twenty four hours after death the figures were respectively 130, 83 and 40 mg per 100 ml. The figures for case 35 are included in table III.

Results in 8 non-diabetic subjects with hyperglycaemia developing in the right side of the heart

In the first case we examined, the blood sugar in the pulmonary artery four hours after death was 290 mg. per 100 ml. and after fifty-one hours it had risen to 610 mg. Similar figures were obtained in seven other non-diabetic subjects. The accompanying figure shows that this rise is confined to blood from the right side of the heart. In cases 18 and 38 the figures obtained soon after death



Comparison of blood sugar content of right and left heart and cerebrospinal fluid.

were within normal limits and a rise was only demonstrated some time later. In case 18 (table II) the rise continued till the third day after death, when no further estimations could be made. In case 38 (table III) the rise was apparent over the first 12 hours after death, but glycolysis was complete at the end of 24 hours.

To exclude the possibility of the increasing amounts of reducing substance in the right side of the heart being associated with protein breakdown, parallel non-protein nitrogen and sugar estimations

were made on blood from the right side of the heart the aorta and the cerebrospinal fluid (table II) The first analysis from case 21

TABLE II

Parallel estimations of sugar and non protein nitrogen in samples of blood and cerebrospinal fluid

Case no	Time after death in hours	Aorta		Right auricle		Cerebro spinal fluid	
		Sugar	Non protein nitrogen	Sugar	Non protein nitrogen	Sugar	Non protein nitrogen
		In mg per 100 ml					
18	8			28	75	15	48
	26			47	84	22	95
	50			100	126	25	123
	73			200	88	35	109
21	5	167	35	147	31	88	34
	30	30	45	83	52	40	50
22	12	39	125	34	105	44	113
	34	44	95	100	171	20	151
19	20			434	87	38	80
20	12	12	50	34	101	13	83
24	18	20	123	40	233	20	147

shows that the non protein nitrogen is practically constant in all three samples, while the sugar varies widely In the second set of samples the non-protein nitrogen has increased and the sugar decreased Examination of the other cases in the table shows still more that protein breakdown bears no relation to the reducing substances in the blood or cerebrospinal fluid Case 18 shows the increase in non protein nitrogen of both blood and cerebrospinal fluid to be closely parallel, while the sugar content is increasing in the blood but glycolysis is complete in the cerebrospinal fluid The slight rise in the reducing substances of the cerebrospinal fluid is probably due to an increase in non glucose reducing substances as shown below

The next step was to prove that the reducing substance present was glucose This was done by yeast fermentation Table III shows that in those cases where high values for reducing substance were obtained in the blood it was mostly glucose These figures are assembled in the columns headed "Glucose" The cases were all non diabetic, yet in five of them the blood in the right side of the heart shows a sugar concentration of over 200 mg per 100 ml In case 35 (multiple fractures of the skull) only is there a high sugar concentration in the cerebrospinal fluid and this may have been due to a heavy contamination with blood, although in case 24, in

TABLE III

Determination of amount of glucose (mg. per 100 ml.) in various samples by fermentation

Case no.	Hours after death	Before fermentation				After fermentation				Glucose			
		C.S.F.	Aorta	Pulmonary artery	Right auricle	C.S.F.	Aorta	Pulmonary artery	Right auricle	C.S.F.	Aorta	Pulmonary artery	Right auricle
30	24	30	39	63	32	19	19	19	21	11	20	44	11
	48	43	30	..	93	40	21	...	26	3	9	...	67
31	24	21	...	107	...	15	.	32	...	6	...	75	...
32	18	19	35	28	22	14	31	23	20	5	4	5	2
	40	42	58	53	51	26	52	32	47	16	6	21	4
33	22	19	28	18	40	16	18	16	25	3	10	2	15
34	26	23	150	273	408	21	25	20	25	2	125	253	383
	48	20	130	272	191	15	29	35	39	5	101	237	152
35	15	179*	...	466	...	16*	...	37	...	163*	...	429	...
	20	120*	342	416	454	14*	34	42	47	108*	308	374	407
36	30	27	24	25	21	19	16	20	20	8	8	5	1
37	35	19	30	52	316	16	14	14	33	3	16	38	283
38	4	43	55	142	105	18	22	18	19	25	33	124	86
	9	27	45	426	248	17	20	31	19	10	25	395	229
	26	18	20	23	73	17	14	21	25	1	6	2	48

* C.S.F. heavily bloodstained.

which the cerebrospinal fluid was similarly contaminated, glycolysis was complete in blood and cerebrospinal fluid when the first estimation was made 18½ hours after death. The non-glucose reducing substances (shown in the columns headed "After fermentation") are present in larger amounts after death, so that their concentration rises slightly. The lowest recorded was 9 and the highest 47 mg. per 100 ml. The average for the cerebrospinal fluid was 13.9, for the aorta 29.2 and for the right heart 21.9 mg. per 100 ml.

In a further series of six cases, including one diabetic, the sugar content of the blood in both sides of the heart and of the liver were determined simultaneously (table IV). It will be seen that high blood sugar values in the right auricle occur only when the liver shows a similar high value.

TABLE IV
*Simultaneous estimation of sugar in the liver
and right and left sides of the heart*

Case no	Hours after death	Sugar content in mg per 100 ml		
		Aorta	Right auricle	Liver
42	5	9	9	35
45	11	19	18	46
39	19	28	90	648
44	36	98	342	572
46	23	75	444	448
43	20	241	390	640
(diabetic)	36	233	480	406
	43	225	344	322
	60	446	378	380

After death, glycogen disappears rapidly from the liver with the formation of glucose, and it seems reasonable to suppose that this glucose might diffuse from the hepatic veins through the short length of the inferior vena cava and appear in the right auricle. In this way high blood sugar values would be obtained in the right heart in cases with a high liver glycogen content at death, while in those cases with a depleted liver glycogen content, such a change would not be shown.

The results shown in table IV agree with this argument and account for the large differences in the blood sugar content of the right and left heart in some non-diabetics. The fermentation experiments show that it is glucose which is responsible for the rise, and the non-protein nitrogen estimations show that the increase in glucose does not run parallel with proteinolysis. The other obvious source would be from the glycogen of the heart muscle, but as we have not been able to demonstrate a similar rise in the left side of the heart, where there is more muscle and consequently more glycogen, this seems highly improbable.

Glycolysis does occur in these cases but its onset seems to be delayed. In case 9 the blood sugar in the pulmonary artery remained at the same level (250-300 mg. per 100 ml.) for three days but at the end of five days it had practically completely disappeared. In case 38 it rose from 140 to 425 mg. per 100 ml. in the first nine hours after death, but after twenty-six hours it had almost disappeared. In case 1 it increased from 250 to 450 mg. per 100 ml. in 51 hours, after which time no further estimations could be made. A possible explanation of this fact is that the normal glycolytic process was masked by the diffusion of glucose produced in the liver.

Results in 6 diabetics

In subjects developing hyperglycæmia in the right heart *post mortem* we have shown that the blood sugar in the left heart is normal. In diabetics, as would be expected, high values are found in both sides of the heart and in the cerebrospinal fluid. These results are shown in table V. It will be seen that glycolysis does

TABLE V
Post-mortem glycolysis in diabetic subjects

Case no.	Time after death in hours	Sugar in mg. per 100 ml.		
		Right auricle	Aorta	Cerebrospinal fluid
7	1	412	...	270
	24	472
	32	464
	50	400	...	90
12	2	226
	13	470	410	70
	32	480	425	128
17	10	560	...	360
43	20	390	241	192
	36	480	233	172
	43	344	225	152
	60	378	446	149
48	4	494	496	486
	7½	684	652	520
	24	748	624	536
50	Capillary blood sugar 4 hours before death 360 mg. per 100 ml.			
	14	378	380	370

occur in the cerebrospinal fluid, but it proceeds more slowly than in non-diabetic cases. In the blood there is little or no reduction in the sugar content and it may even increase in the left heart as well as in the right. In case 43 (table IV) there is increasing hyperglycæmia in the right heart while the liver sugar remains high and

this probably occurs in all diabetics with a high liver glycogen content at death. The increase in the sugar content of the left heart is also shown in case 43 (table V)

Glycolysis in vitro

In vitro glycolysis experiments were carried out with diabetic blood, normal blood and normal blood with added dextrose. Minimal quantities of potassium oxalate or heparin were used as anticoagulants. The temperature at which the experiments were carried out was recorded and the results are shown in table VI.

TABLE VI
Glycolysis in vitro

Heparin as anticoagulant				Oxalate as anticoagulant			
Case	Time in hours	Temp	Mg. glucose per 100 ml	Case	Time in hours	Temp	Mg. glucose per 100 ml
Diabetic	0	18° C	132	Diabetic	0	22° C	214
	5½		115		1½		187
	24		92		5		190
	30		54		18		180
Diabetic	0	18° C	278		24		164
	6		125		40		158
					96		154
Normal (added dextrose)	0	20° C	384	Normal (added dextrose)	0	20° C	440
	17		340		3		420
	24		318		24		354
	41		290	Normal	0	20° C	86
Normal (added dextrose)	0	20° C	990		3		76
	6		980		24		32
	23		943	Normal	0	20° C	112
	28		860		3		101
Normal	0	20° C	137		24		51
	17		46				
	24		38				
	41		11				

Study of the figures shows that the amount of sugar undergoing glycolysis in the first 24 hours is roughly comparable with all three types of blood. The figures in mg per 100 ml for heparin are 40, 66, 47 and 99, and for oxalate 50, 86, 54 and 61. These experiments also rule out the possibility of a high concentration of glucose being inhibitory to the glycolytic enzyme, a fact which might have accounted for the diminished or absent post mortem glycolysis in diabetic blood in the cadaver. According to these figures oxalate has no more effect in delaying glycolysis than heparin.

DISCUSSION

These investigations have brought out certain points which must be observed if hyperglycæmia or hypoglycæmia, present during life, is to be diagnosed after death. Blood from the left heart must be used for investigation, as post-mortem hyperglycæmia may occur in the right heart of subjects with a normal blood sugar content during life. The time after death at which the blood is collected must be noted. Although we have had no cases of hypoglycæmia it seems probable that estimations must be made within two hours of death to be reasonably sure that it did exist, as normal cases may show complete glycolysis after three and a half hours.

Blood sugar values over 200 mg. per 100 ml. in the left heart and correspondingly high values in the cerebrospinal fluid after death are constantly found in patients who have had hyperglycæmia. In these cases no appreciable glycolysis has been noted in the blood, even up to 54 hours after death. Glycolysis in the cerebrospinal fluid does occur but proceeds much more slowly than in normal subjects. In subjects with a normal blood sugar content glycolysis proceeds rapidly and is complete in from $3\frac{1}{2}$ to 7 hours. In-vitro experiments show no such difference between hyperglycæmic and normal blood.

Hyperglycæmia may be due to causes other than diabetes, of which raised intracranial tension is the most likely. These, of course, must be excluded.

CONCLUSIONS

1. Hyperglycæmia may be diagnosed if a blood sugar value of 200 mg. per 100 ml. or more is found after death in the left heart.
2. Hyperglycæmia may develop in the right heart of non-diabetic subjects (8 out of 42 cases).
3. Hypoglycæmic coma as a cause of death cannot be confirmed unless the blood is examined within 2 hours of death.
4. Diabetic blood glycolyses more slowly in the cadaver than *in vitro*.

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THE BACTERICIDAL ACTION OF NORMAL SERA

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IN a previous publication (Gordon and Hoyle, 1936) it was shown that absorption of normal guinea-pig serum with large amounts of heated suspensions of certain bacteria caused a reduction in its bactericidal power for all organisms tested, but especially for the absorbing organism. Excessive absorption, however, tended to remove the complement and so to destroy all bactericidal activity. This specific absorption could be shown to occur not only with organisms of different species such as *B. typhosus*, *B. paratyphosus* A, *B. dysenteriae* (Shiga), *B. dysenteriae* (Flexner), the meningococcus and *M. catarrhalis*, but also with different strains of the two last-named. It was thought that the specific effect was due to the introduction into the serum of a specific growth-promoting factor derived from the absorbing organism. In the investigation reported in the present paper it has been found that no such specific growth-promoting factor is added to the serum. The specific removal of bactericidal power indicates either (a) that there are in normal serum not only many species-specific antibodies (Mackie and Finkelstein, 1931), but also a vast number of strain-specific antibodies, or (b) that absorption has some specific effect on a general bactericidal antibody.

Experimental methods

Twelve agar slopes in tubes 1 inch in diameter were inoculated with each of three strains of *M. catarrhalis*—"O," "3" and "R.H.S."—and were incubated at 37° C. for 48 hours. The growths were washed off with sterile distilled water, killed by heating at 60° C. for 30 minutes and centrifuged, the sediments being washed with sterile distilled water and each finally resuspended in 1.0 ml. of distilled water to produce very dense suspensions. Twelve ml. of sterile guinea-pig serum were separated from blood obtained by heart puncture and divided into four quantities of 3.0 ml. To each of three of these was added one of the three suspensions and to the fourth 1.0 ml. of sterile distilled water. These were kept at 0.5° C. for 14 hours with frequent agitation. The sera were separated by centrifugation and together with the normal serum were heated at 55° C. for 30 minutes to destroy complement. Each heated serum was divided into three quantities of 1.0 ml., which were then inoculated with 0.1 ml. of faintly opalescent live

suspensions of the three strains O, 3 and R.H.S. in distilled water. Viable counts were made at once and after 3, 6, 9 and 12 hours' incubation at 37° C. by transferring loopfuls of dilutions 1:100 and 1:1000 of the inoculated sera in distilled water to heated blood agar plates and counting the resulting colonies after incubation for two days at 37° C.

The results are shown in table I. There is no evidence of strain-specific growth-promoting factors in the sera absorbed with strains of *M. catarrhalis*.

TABLE I

The effect of absorption with M. catarrhalis on the growth-promoting properties of heated serum for various strains of that organism

Absorbing strain		O			3			R.H.S.			Nil		
Strain inoculated		O	3	R.H.S.	O	3	R.H.S.	O	3	R.H.S.	O	3	R.H.S.
Time (hrs.)	Dilution of sera												
0	1:100	41	45	85	62	52	+	47	51	79	57	73	+
	1:1000	12	5	28	7	10	21	13	4	14	2	11	17
3	1:100	54	57	64	42	66	52	91	60	87	76	66	69
	1:1000	18	9	9	12	21	5	15	5	19	12	15	13
6	1:100	+	+	+	+	78	86	+	+	+	+	+	+
	1:1000	30	10	19	50	12	29	58	34	57	14	19	31
9	1:100	+	+	++	++	+	++	+	+	++	++	+	++
	1:1000	90	20	+	+	25	31	29	71	78	43	59	+
12	1:100	++	++	++	++	++	++	++	++	+++	++	++	++
	1:1000	78	98	+	+	+	+	+	+	++	+	+	+

In all tables ++++ = confluent growth; +++ = very heavy growth; ++ = heavy growth; + = 100-200 colonies.

Figures = number of colonies <100.

Bactericidal specificity for three strains of M. catarrhalis

Bactericidal specificity was demonstrated in guinea-pig sera absorbed with the three strains of *M. catarrhalis* by a technique similar to that described above except that the sera were not heated. Quantities of 0.1 ml. of suspensions of the three strains in distilled water containing 100 million organisms per ml. were used as inocula and subcultures were made from the undiluted sera. The results are shown in table II. It is clear that the bactericidal power of an absorbed serum, while generally diminished for all three strains, is also specifically diminished for the absorbing strain.

Attempts were made to remove the complement by absorption with very large amounts of heat-killed suspensions of *M. catarrhalis*. It was found that while no specific growth-promoting effect was obtained in those cases in which removal of the complement was

complete, in other cases where sufficient complement remained to enable bactericidal action to take place the effect of absorption was relatively specific.

TABLE II

The bactericidal action of absorbed and unabsorbed sera for M. catarrhalis

Absorbing strain	O			3			RHS			XII		
Strain inoculated	O	3	RHS	O	3	RHS	O	3	RHS	O	3	RHS
Time (hrs.)												
0	+++	++++	+++++	+++	++++	+++++	+++	++++	+++++	+	+++	+++
1	+++	+++	+	1	++++	95	0	+++	+++++	0	0	0
2	+++	++	0	0	++++	14	0	++	+++++	0	0	0
4	+++	+	3	0	++++	2	0	+	+++++	0	0	0
6	+++	20	2	0	++++	0	0	14	+++++	0	0	0

* Organisms killed before subculture could be carried out

The growth-promoting properties of guinea-pig sera absorbed with various organisms

Gordon and Hoyle showed that a similar general and specific diminution in the bactericidal action of guinea-pig serum was obtained after absorption with *V. cholerae*, *B. dysenteriae* (Flexner), *B. dysenteriae* (Shiga) and *B. typhosus*. Experiments were carried out to determine whether this specificity was related to the addition of growth-promoting factors during the process of absorption. The technique was the same as for *M. catarrhalis* except that *B. dysenteriae* (Flexner), *B. dysenteriae* (Shiga) and *B. typhosus* were killed by heat at 65° C. for one hour and *V. cholerae* at 60° C. for 1 hour to avoid the formation of large and not readily dispersible bacterial clumps. The sera were heated at 55° C. for 30 minutes to destroy complement. The results are shown in table III. Again there is no evidence of specific growth-promoting factors in sera after absorption with these organisms, but there is a slight non-specific growth-promoting effect.

The bactericidal action of guinea-pig, rabbit and human sera on a series of organisms and its relation to complement activity

To determine whether various sera differed in bactericidal antibody content apart from variation in complement titre, unheated guinea-pig, rabbit and human sera were tested against the gonococcus, *M. catarrhalis*, *B. typhosus*, *B. paratyphosus* A,

TABLE III

The effect of absorption of guinea-pig serum with various organisms on the growth-promoting properties of the heated serum for these organisms

Absorbing strain		<i>V. cholerae</i>				<i>B. dysenteriae</i> (Flexner)				<i>B. dysenteriae</i> (Shiga)				<i>B. typhosus</i>				XII			
Time (hrs.)	Organism inoculated	C	F	S	T	C	F	S	T	C	F	S	T	C	F	S	T	C	F	S	T
		Dilution of sera																			
0	1:100	1	4	8	11	0	3	3	14	0	3	4	12	0	7	10	10	0	4	9	18
	1:1000	0	1	0	0	0	1	2	3	0	3	0	3	0	2	1	2	0	1	1	2
5	1:100	1	30	+	+	2	+	++	+	6	+	+	++	1	+	++	++	3	31	47	+
	1:1000	0	4	21	21	0	33	+	15	0	15	20	85	0	37	98	+	0	6	4	8
9	1:100	+	++	++	++	+	++	++	++	+	++	++	++	+	++	++	++	68	+	++	++
	1:1000	14	+	++	++	61	++	++	++	2	73	++	++	17	++	++	++	1	30	++	+

C = *V. cholerae*; F = *B. dysenteriae* (Flexner); S = *B. dysenteriae* (Shiga); T = *B. typhosus*.

B. dysenteriae (Shiga), *B. dysenteriae* (Flexner) and *V. cholerae*, using the technique previously described for *M. catarrhalis*. The complement titre of the guinea-pig serum was high, of the human serum low and of the rabbit serum very low. The results (table IV)

TABLE IV

The bactericidal action of guinea pig, rabbit and human sera on various organisms

Time (hrs)	Organism inoculated						
	<i>Cono-coccus</i>	<i>M. catarrh-alis</i>	<i>B. typhosus</i>	<i>B. para-typhosus A</i>	<i>B. dysenteriae</i> (Shiga)	<i>B. dysenteriae</i> (Flexner)	<i>V. cholerae</i>
Guinea-pig							
0	+++	++++	++++	++++	+++	++++	++++
1	0	0	++++	+++	++	+++	++++
2	0	0	++++	+	+	+++	++++
4	0	0	++	?	2	+	8
6	0	0	17	1	40	42	0
Rabbit							
0	9	++++	++++	++++	++++	++++	++++
1	0	0	+++	2	+	+++	2
2	0	0	++++	0	23	++	0
4	0	0	++	0	9	+	0
6	0	0	+	0	0	22	0
Human							
0	+++	++++	++++	++++	+++	++++	++++
1	+++	0	++++	0	5	+++	++++
2	++	0	+++	1	0	++	++++
4	+	0	20	0	0	+	++++
6	0	0	7	0	0	44	++++

show the low bactericidal action of the human serum on the gonococcus whereas the guinea pig serum with a higher and the rabbit serum with a lower complement titre were both markedly bactericidal, most of the organisms were killed by the rabbit serum before the initial subculture could be made. In this experiment the human serum had no bactericidal action on *V. cholerae*, but another human serum killed *V. cholerae* in one hour. For this organism again the rabbit serum was the most bactericidal. It is clear that for the gonococcus and *V. cholerae* there is marked difference in bactericidal activity between various sera which cannot be explained by variation in complement titre.

The bactericidal action of human, guinea pig and rabbit sera on the gonococcus was investigated in greater detail, using graded

inocula prepared by dilution of a bacterial suspension in broth, and was correlated with the complement titres of the sera. The technique was otherwise as for *M. catarrhalis*. The results are shown in table V. The rabbit serum, which had a lower complement titre than the guinea-pig serum, was again the more bactericidal, and inactivation of complement completely destroyed the bactericidal action of both sera. The human serum was much the least bactericidal, while its complement titre was comparable with that of the rabbit serum. After inactivation of complement the human serum alone showed a slight bactericidal action for the most dilute bacterial suspension. Heavier inocula masked this effect. Similar results were obtained with several strains of the gonococcus and several human sera.

Because of the differences noted above in the action of two human sera on *V. cholerae*, six human sera were tested against five strains of that organism, using the standard technique described for *M. catarrhalis*. The results (table VI) show that three sera were markedly bactericidal, all five strains being rapidly killed. Of the three remaining sera, one (Anderson) was rapidly bactericidal for strain Ogawa, whereas two (Stewart and Sturgess) were not. The strains Zeiss and Jenkins were very susceptible to the bactericidal action, they alone being killed after three hours by the three less powerful sera (Stewart, Anderson, Sturgess). The complement titres of five of the sera were closely similar, while that of Stewart, one of the least bactericidal, was much higher. Similar results were obtained on another occasion with sera from the same donors. Six other human sera also showed differences in bactericidal action and in the susceptibility of the strains, Zeiss and Jenkins being the most susceptible.

Summary

The above results have again shown that absorption of a normal serum with a series of strains of one organism causes a general diminution in bactericidal power for all the strains, but there is a more striking diminution for the strain with which the serum was absorbed. It has been demonstrated that no specific growth-promoting factor is introduced in this process, contrary to the suggestion made by Gordon and Hoyle. Two possibilities remain. Either there are in normal serum not only many species-specific antibodies, but also a vast number of strain-specific antibodies capable of individual absorption, or there is a general bactericidal antibody which can be so modified by contact with a large excess of any particular organism or strain as to render it specifically inactive for that organism or strain.

Further evidence of the variation and apparent multiplicity of antibodies in normal sera is afforded by the results obtained

TABLE V—The bactericidal action of human, guinea pig and rabbit sera on the gonococcus

Undiluted sera															Heated sera							
Human															Guinea pig						Rabbit	
Human															Guinea pig						Rabbit	
Human															Guinea pig						Rabbit	
Human															Guinea pig						Rabbit	
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with *V. cholerae* and the gonococcus. The latter show that while the bactericidal action of normal human sera on the gonococcus is relatively weak as compared with that of guinea-pig and rabbit sera, it is not entirely absent, as stated by Abdoosh (1936).

The methods employed would appear to be suitable for determining antigenic differences between strains of certain organisms susceptible to bactericidal action.

The strains used in this work, with the exception of those of the gonococcus, were obtained from the National Collection of Type Cultures. We should like to express our gratitude to the Medical Research Council for a grant to one of us (J. G.) in aid of expenses.

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THE EFFECT OF A CO₂ ATMOSPHERE ON BLOOD CULTURES *

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IN a series of 64 routine blood cultures, 64 samples from 59 patients were seeded in triplicate into 0.05 per cent. liquid broth and, in 1 c.c. quantities, into heart-extract agar at 42° C., which was immediately poured into plates. Each set was incubated aerobically, anaerobically and in air containing 5 per cent. CO₂. There were thirteen positive cultures, which are listed in table I. It will be

TABLE I

Growth of organisms in blood cultures in different atmospheres

Organisms recovered	Result of cultivation		
	aerobically	in air containing 5 per cent. of CO ₂	anaerobically
Anaerobic streptococcus	—	—	+
<i>Bacteroides</i>	—	—	+
<i>Br. abortus</i>	—	+	+
<i>Haemophilus</i> n. sp.	(+)	+	+
<i>Str. pneumoniae</i> (group IV) 1	—	+	+
<i>Str. pneumoniae</i> (group IV) 2	+	+	+
<i>Str. viridans</i> (4 strains)	+	+	+
<i>Str. pyogenes</i> type XVI	+	+	+
<i>Staph. aureus</i>	+	+	+
<i>Bact. coli</i>	+	+	+

(+) = films positive, aerobic subcultures negative.

seen that all the aerobes grew in the CO₂, but that two, *Brucella abortus* and *Str. pneumoniae* 1, grew in CO₂ but not in air. The effect with *Br. abortus* is to be expected. The failure of the *Str. pneumoniae* to grow aerobically was probably a chance effect, since the organisms in the circulating blood were scanty, as judged by the absence of colonies from the three plates; moreover, the growth of the strain was shown by subsequent quantitative tests to be unaffected by 5 per cent. CO₂ in air.

* This work formed part of a thesis for the Ph.D. degree of the University of London.

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But for the routine incubation in CO_2 , a new species of *Hæmophilus* (Khairat, 1940) would not have been isolated, for although the organism grew in the blood culture tube incubated aerobically and could be demonstrated therein by means of films, subcultures on blood agar were sterile except in an atmosphere of 5 per cent. CO_2 in air.

There can be no doubt of the value of culture in CO_2 as an addition to routine aerobic and anaerobic culture of blood samples, but the triplication of culture tubes is laborious and means that only one third of the venous sample is available for each atmosphere. The object of this investigation was to determine quantitatively the effect of 5 per cent. CO_2 on the aerobic growth of organisms encountered in blood culture, with a view to omitting the aerobic cultures and cultivating one half of the venous sample in CO_2 and one half anaerobically. The organisms were tested on plates of 5 per cent. horse blood agar and in tubes of 5 per cent. horse blood broth, the conditions in the latter simulating those in a routine blood culture tube sufficiently closely for the purposes of the experiment. The effect of CO_2 was measured by comparison of colony counts and of colony size on the plates, and of the dilutions of bacterial suspension growing in blood broth.

Method

A 24-hour broth culture was vigorously shaken for at least two minutes to break up aggregates. The degree of dispersion was checked by examination of a hanging-drop, and the culture was diluted with broth until its turbidity was just visible. The resulting suspension contained about 10^6 - 10^7 viable organisms per c.c. Accordingly, tenfold dilutions from 10^{-3} to 10^{-8} were seeded on to the solid media in 0.02 c.c. volumes. Eight plates were inoculated by the "surface-viable" counting technique of Miles and Misra (1938) and incubated at 37°C ., four in air and four in air containing 5 per cent. of CO_2 . In addition, a standard inoculum of 0.02 c.c. and one of either 0.06 or 0.08 c.c. were each added to two tubes containing 5 c.c. of blood broth, making two sets of fluid subcultures for incubation in the two atmospheres. Results were read after 24 hours, or after 48 hours with more slowly growing organisms. Colony diameters were measured micrometrically on discrete colonies separated from their nearest neighbours by more than one colony diameter, and the mean and standard deviation of 15-20 diameters determined. In the fluid media growth turbidity was in all cases checked by seeding loopfuls on to two blood agar plates which were incubated in air and in air+ CO_2 respectively. The results of the broth tests were recorded as the maximum dilution producing growth. For this purpose, the inocula of 0.06 and 0.08 c.c. were regarded as standard inocula (0.02 c.c., $\times 3$ and 4), and interpolated in the 10-fold dilution series thus:— $10^{-5.5}$, 10^{-6} , $10^{-6.5}$, etc., and $10^{-5.3}$, 10^{-6} , $10^{-6.3}$ etc.

The strains of bacteria, unless otherwise stated, were freshly isolated from human tissues or blood and in most cases the first subculture was used for the test. All the stock strains employed were fully "smooth."

The organisms were *Str. viridans* 1 and 2, from bacteraemias; *Str. pyogenes* type II, from the throat of a scarlet fever patient; *Str. pneumoniae*

group IV 2 and 3, from bacteræmias, *Str faecalis*, from faeces, *Staph aureus* 1 and 2, from bacteræmias, *Bact coli* 1, from a bacteræmia, *Bact coli* 2, from faeces, *Bact typhosum*, from human spleen *post mortem*, *Bact paratyphosum* B, recently isolated from a bacteræmia, *Bact enteritidis*, from the gall bladder of a guinea pig, *Br melitensis*, from a bacteræmia (primary culture used), *Br suis* 1 and 2, stock strains, *H influenza*, recently isolated from naso pharynx, *H para influenza* stock strain 441, from an endocardial vegetation, a new species of *Hæmophilus*, from blood and endocardial vegetations, *Proteus vulgaris*, from human liver *post mortem*, a Gram positive micrococcus, from an endocardial vegetation, *Ps pyocyanea*, and *Staph albus*. Strains of organisms known to be benefited by CO₂, namely *Br abortus*, *Neisseria gonorrhææ* and *N meningitidis* were not included.

Results

Table II summarises the results. The significance of the differences between each pair of plate counts and between each pair of mean colony diameters was estimated in the usual way, using "Student's" *t* and taking *P* as 0.01 (Fisher, 1936).

The counts recorded in columns 2 and 3 indicate the effect of CO₂ on the germination of bacteria. Of the 23 organisms tested, germination was definitely enhanced only with the freshly isolated *Str pneumonia* 3, and with the new *Hæmophilus*. The differences between the remaining pairs of counts are not significant. Nor is there among these any bias in favour of CO₂ action, for of these insignificant differences 12 are positive and 9 negative.

The growth rate of colonies from the germinating organisms is indicated in columns 4 and 5. CO₂ favoured the colony growth of *Str pneumonia* 2, the new *Hæmophilus* and the micrococcus. There was a less definite favourable action on *Bact coli* 1 and an equally indefinite depressant action on *Bact typhosum* and *H influenza*. The remaining differences are insignificant, 10 favour CO₂, 2 are neutral and 5 are unfavourable.

Except in the case of the new species of *Hæmophilus*, where the CO₂ definitely enhances growth, the differences between the maximum dilutions germinating in fluid culture are insignificant. An unwieldy number of tubes would be required for each culture dilution before the comparison would yield a dependable answer (see Fisher, 1936, section 17). Of such differences as there are, 9 favour CO₂ and 6 favour growth in air. 8 are neutral.

Discussion

The presence of 5 per cent of CO₂ apparently does not inhibit the germination of any of the organisms tested. In certain cases germination is favoured, in the remainder, any indication of inhibition or stimulation by CO₂ cannot be assigned with confidence to any cause other than the operation of chance. The results confirm

TABLE II

Germination and growth of bacteria in air and in air+5 per cent. of CO₂

Organism tested	Solid media				Fluid media	
	Number of colonies developing from 0.02 c.c. of original culture		Mean diameter of colonies (mm.)		Dilution of original culture which gave growth	
	Air	5 per cent. CO ₂ in air	Air	5 per cent. CO ₂ in air	Air	5 per cent. CO ₂ in air
<i>Str. viridans</i> 1 . . .	950,000	1,070,000	0.40	0.45	10 ⁻⁶	10 ^{-6.5}
" " 2 . . .	180,000	166,000	0.28	0.28	10 ⁻⁶	10 ^{-5.5}
<i>Str. pyogenes</i> type II .	670,000	720,000	0.76	0.73	10 ^{-5.3}	10 ^{-5.3}
<i>Str. pneumoniae</i> group IV 2	2,000,000	1,660,000	0.60	0.62	10 ⁻⁷	10 ^{-6.3}
" " " " 3	<u>60,000</u>	<u>1,900,000</u>	<u>0.42</u>	<u>1.65</u>
" " " " 3	1,470,000	2,000,000	0.65	0.76	10 ^{-6.5}	10 ^{-6.5}
5th subculture						
<i>Str. pneumoniae</i> , group IV 3	850,000	1,070,000	0.67	0.77	10 ⁻⁶	10 ⁻⁶
7th subculture						
<i>Str. faecalis</i> . . .	257,000	247,000	0.47	0.45	10 ⁻⁵	10 ⁻⁵
<i>Staph. aureus</i> 1 . . .	2,800,000	3,250,000	0.81	0.77	10 ⁻⁶	10 ^{-6.3}
" " 2 . . .	4,660,000	7,330,000	1.77	1.93	10 ⁻⁶	10 ⁻⁵
<i>Bact. coli</i> 1 . . .	4,300,000	4,830,000	1.15	1.31	10 ⁻⁶	10 ^{-6.5}
" " 2 . . .	14,300,000	19,000,000	2.03	1.98	10 ⁻⁷	10 ⁻⁸
<i>Bact. typhosum</i> . . .	850,000	925,000	1.58	1.24	10 ⁻⁶	10 ⁻⁶
<i>Bact. paratyphosum B</i> .	1,050,000	825,000	1.67	1.78	10 ^{-6.5}	10 ^{-7.5}
<i>Bact. enteritidis</i> . . .	315,000	345,000	1.78	2.20	10 ^{-5.5}	10 ⁻⁶
<i>Br. melitensis</i> . . .	440,000	410,000	0.23	0.33	10 ^{-5.5}	10 ^{-5.5}
<i>Br. suis</i> 1 . . .	1,770,000	1,900,000	0.64	0.68	10 ⁻⁷	10 ^{-6.3}
" " 2 . . .	2,350,000	3,100,000	0.48	0.60	10 ⁻⁷	10 ⁻⁶
<i>H. influenzae</i> . . .	850,000	800,000	1.00	0.83	10 ^{-5.5}	10 ⁻⁶
<i>H. parainfluenzae</i> . . .	1,200,000	1,050,000	0.46	0.62	10 ⁻⁵	10 ⁻⁶
<i>Hæmophilus</i> n. sp. bacteriæmia strain	<u>240,000</u>	<u>24,000,000</u>	<u>0.55</u>	<u>0.78</u>	10 ^{-4.3}	10 ⁻¹⁰
<i>Hæmophilus</i> n. sp. vegetation strain	<u>50</u>	<u>2,950,000</u>	<u>0.56</u>	<u>1.23</u>	10 ⁰	10 ⁻³
<i>Proteus vulgaris</i> . . .	5,700,000	4,800,000	0.71	0.71	10 ^{-7.3}	10 ^{-7.3}
Micrococcus . . .	280,000	272,500	0.43	0.84	10 ⁻⁴	10 ⁻⁴
<i>Ps. pyocyanea</i> . . .	37,000	53,000	2.14	1.57	10 ⁻⁵	10 ^{-5.5}
<i>Staph. albus</i> . . .	116,000	153,000	0.32	0.46	10 ^{-6.5}	10 ^{-5.5}

Figures in heavy type and underlined differ by a statistically significant amount; those in heavy type only by an amount of doubtful significance.

those of Valley and Rettger (1927), who found that concentrations of CO₂ up to 50 per cent. failed to inhibit the growth on solid media of stock strains of *Str. pyogenes*, *Bact. coli*, *Bact. typhosum*, *Bact. paratyphosum A* and *B*, *B. anthracis* and *V. cholerae*.

Five per cent. CO₂ enhanced the colony growth-rate of four organisms and depressed that of two (*Bact. typhosum* and *H. influenzae*), but in neither of these cases was the depression associated with any inhibition of germination.

The experimental evidence at least does not contra-indicate the omission of routine aerobic cultivation and the substitution of cultivation in CO₂. There are several considerations, however, which preclude its immediate application. Topley and Wilson (1936) state that some strains of *Br. suis* are inhibited by CO₂, and base their statement on a roughly quantitative study of a number of strains (Prof. G. S. Wilson, personal communication). It was not possible to obtain freshly isolated *suis* strains. The two stock strains tested were slightly benefited by CO₂. Unpublished studies in this laboratory show that the CO₂ requirements of a number of organisms are rapidly and sometimes profoundly modified by subculture on stock media, as is shown by the effect of subculture on *Str. pneumoniae* 3 (table II). Arguments, therefore, from the behaviour of stock strains are not dependable, since it is the primary isolation of organisms in bacteriæmia that is under consideration. Moreover, it is obvious from the different effects of CO₂ on two strains of the same organism (*Str. pneumoniae* 2 and 3, *Staph. aureus* 1 and 2, *Bact. coli* 1 and 2—table II) that the 23 strains tested cannot be said necessarily to exhibit the typical CO₂ sensitivity of the species they represent.

Culture in CO₂ is as necessary as anaerobic culture for blood samples from patients with a suspected bacteriæmia; in the short series of cultures reported it resulted in the discovery of a new species of *Hæmophilus* and of an unsuspected infection with *Br. abortus*. Whether it can safely take the place of aerobic culture must await quantitative tests on a larger number of strains, especially of *Br. suis*, and large-scale comparison of blood cultures in the two atmospheres. It is suggested, however, that if the choice lies between culture in air and in CO₂, the evidence justifies the use of the latter, i.e. incubation in air containing 5-10 per cent. of CO₂.

Summary

The germination and growth rate of 21 freshly isolated strains of pathogenic bacteria and of 2 stock strains of *Br. suis* were tested under aerobic conditions and in the presence of CO₂ with a view to substituting incubation in air *plus* 5 per cent. CO₂ for incubation in air in routine blood culture work.

Germination of the bacteria was in no case significantly depressed by the presence of CO_2 ; in some cases it was enhanced. The growth rate but not the germination of *Bact. typhosum* and *H. influenzae* was slightly depressed by CO_2 ; that of the remainder was either unaffected or enhanced.

There is evidence that the strains tested do not necessarily represent the CO_2 -sensitivity of the species to which they belong, and though the results emphasise the value of including incubation in air containing CO_2 as a routine in blood culture work, they cannot be said to warrant the omission of aerobic culture.

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ENDOCARDITIS DUE TO A NEW SPECIES OF *HÆMOPHILUS**

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(PLATE LV)

VARIOUS hæmophilic bacilli have been described as responsible for fatal endocarditis in the human subject (Miles and Gray, 1938). The case described below is apparently the first on record due to a hæmophilic bacillus that has the X and V requirements of *H. canis*, i.e. is capable of synthesising V but not X factor.

DESCRIPTION OF CASE

The patient, a female aged 28, was admitted to University College Hospital on 30.7.38 under Dr Kenneth Harris with a history of fever (up to 104° F), generalised muscular pains, headache and vomiting for ten days and of "red" urine for five days. On the 11th day faint presystolic and blowing systolic murmurs, blood and albumen in urine, anaemia, which persisted till death, no leucocytosis. A hæmophilic bacillus was isolated from the blood. The patient remained *in statu quo*, with continued pyrexia and bacteraemia, and was discharged at her own request on the 91st day. She was readmitted in a semi-comatose condition on the 161st day. Two days later bronchopneumonia became manifest and mucopurulent oropharyngitis due to *Staph. aureus*. Death occurred on the 165th day. For two periods chemotherapy was attempted, first with a total of 26.7 g of sulphanilamide, and later with a total of 96 g of 2-sulphanilyl aminopyridine. Both drugs reduced the pyrexia during the period of administration, but the degree of bacteraemia (20-100 organisms per c.c.) was unaffected.

On four occasions during life the hæmophilic bacillus was isolated from the blood. Growth occurred in fluid media incubated in air and in air containing 5 per cent CO₂, but subculture on to solid media was successful only when the plates were incubated in air + 5 per cent CO₂. All four strains were alike morphologically and biochemically. The last two were agglutinated to the same titre by an antiserum prepared against one of them (the first two strains died before the serological tests were made).

Post mortem findings (8 hours after death)

Skin numerous petechiae on abdomen and chest. *Heart* 360 g. Sixty c.c. of slightly turbid pericardial fluid. Mitral valve showed subacute

* This work formed part of a thesis for the Ph.D. degree of the University of London.

† With a grant from Iouad I University, Cairo.

bacterial endocarditis with large, yellowish and friable vegetations, ulceration and scarring of the valve cusps, and ruptured chordæ tendinæ. Right ventricle and both auricles hypertrophied. Microscopically, some miliary embolic infective foci in the myocardium. The vegetations showed a marked inflammatory reaction around large irregular masses of Gram-negative cocco-bacilli. *Aorta*: a large "saddle" embolus at the bifurcation of the abdominal aorta, with recent thrombi extending along each iliac artery (fig. 1). *Lungs*: right 710 g., left 680 g.; very congested and œdematous. Basal bronchopneumonia. About 30 c.c. of fluid in each pleural cavity. Microscopically, patches of bronchopneumonia. *Liver*: 1720 g.; congested and firm. *Spleen*: 380 g.; greatly enlarged, with two old infarcts and several small recent ones. *Kidneys*: both 380 g.; swollen and congested; capsules stripped readily; surface mottled; glomeruli prominent; cortical pattern blurred. Old infarct in left kidney. Microscopically, focal embolic nephritis. *Alimentary canal*: acute inflammation of palate, with a greyish gelatinous membrane, easily detachable; similar exudate in naso-pharynx. Tonsils swollen and ulcerated. *Brain*: 1180 g.; no obvious abnormality.

Post-mortem cultures yielded the following organisms. Heart blood, the hæmophilic bacillus and *Staph. aureus*. Pericardial fluid, *Staph. aureus*. Washings of mitral and aortic vegetation, the hæmophilic bacillus and *Staph. aureus*. Crushed washed mitral vegetation, hæmophilic bacillus in profusion and a few *Staph. aureus*; crushed washed aortic embolus, pure culture of the hæmophilic bacillus. Lung, enlarged bronchial lymph nodes and liver, *Staph. aureus*. Spleen and kidney, both parenchyma and infarcts sterile. Washed and crushed tonsillar tissue, pure culture of *Staph. aureus*.

All the post-mortem strains of the hæmophilic bacillus resembled the blood culture strains morphologically and biochemically and were agglutinated to full titre by a serum prepared against a blood culture strain. That the bacillus was the cause of the disease is shown by the fact that it was grown from the blood stream on four occasions, 153, 146, 123 and 86 days before death, from the blood after death, in pure culture from the crushed mitral vegetation after its surface had been freed from blood by thorough washing, and from the embolus at the bifurcation of the aorta after similar treatment. The *Staph. aureus*, which produced plasma-coagulase and a toxin, appears to have been responsible for a terminal bacteriæmia with bronchopneumonia. It was isolated two days before death in large numbers from the thick brown pharyngeal coating that accompanied the sore throat and in pure culture, *post mortem*, from lungs, bronchial glands, pericardial fluid and liver; also from the blood in mixed culture with the bacillus. Wright (1925), Burn and Harvey (1936) and Miles and Gray (1938) record cases with bacteriæmia in life, where an additional organism was isolated *post mortem*.

SYSTEMATIC DESCRIPTION OF THE HÆMOPHILIC BACILLUS

Morphology on blood agar after 24 hours at 37° C. in air containing 5 per cent. of CO₂. When freshly isolated, the organism is mainly cocco-bacillary with a few short rods and dumb-bell forms ($1.5-2 \times 0.4 \mu$) with parallel sides, round ends and straight axes, arranged singly and in irregular clumps

NEW SPECIES OF *HÆMOPHILUS*

FIG. 1.—“Saddle” colony, *H. aphrophilus*, with recent thrombi propagated

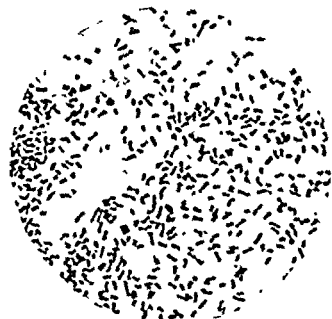


FIG. 2.—*H. aphrophilus* on fresh isolation. Blood agar; 24 hours at 37° C. in air containing 5 per cent CO_2 , $\times 1500$



FIG. 3.—*H. aphrophilus* after repeated subculture. Blood agar; 24 hours at 37° C. in air containing 5 per cent CO_2 , $\times 1500$.

(fig. 2). After repeated subculture, the modal form is a long bacillus with bent axis; occasional filaments occur (fig. 3). In fluid media it is arranged mainly in clumps. Non-motile, non-sporing, Gram-negative, non-acid-fast.

Growth on 5 per cent. horse blood agar. One day at 37° C. in air containing 5 per cent. of CO₂: circular entire-edged colonies, convex, 0.15-0.4 mm., translucent, with smooth glistening surface; homogeneous structure, butyrous consistency, emulsifying easily. Three days: 0.5-0.7 mm., slightly yellowish; round confluent growth, with olive-green discolouration of the blood agar; no hæmolytic.

Variant colonies. 1. "*Mulberry*" form. A few colonies only on fresh isolation on blood but not on Fildes's agar. Dome-shaped, with the surface made up of tightly packed small protuberances like that of a mulberry. The colony burrows into the medium. Broth cultures are very granular, with numerous colonies sticking to the glass. Subcultures yield only the smooth convex colony and "minute smooth convex" (types 2 and 3). Suspensions very unstable. Morphologically, cocco-bacilli predominate. 2. "*Smooth convex*" form. The usual form obtained either on fresh isolation or on subculture. Yields the same type on subculture and occasionally "minute smooth convex" colonies, but never the "mulberry" form. Less granular growth in broth. 3. "*Minute smooth convex*" form. Occasionally seen, usually yields the "smooth convex" form and never the "mulberry." The smooth convex and the minute forms are less unstable in suspension and more bacillary in morphology than the mulberry form. It seems that the mulberry form is equivalent to an unstable S form.

Tryptic digest broth. Two days at 37° C. in air. Slight growth, faint uniform turbidity and small (0.15-0.2 mm.) discrete colonies sticking to the glass. No surface film, but a slight ring forms after 3-4 days' incubation. The discrete colonies that stick to the glass are most frequently found on fresh isolation or in glucose phosphate broth.

Gelatin stab. No growth in 30 days at 20° C. in air or air containing 5 per cent. of CO₂. After 4 days at 37° C. in air, slight growth without liquefaction.

Metabolic requirements. On solid media grows best in air containing 5 per cent. of CO₂. Poor anaerobic growth. No growth in air unless very heavy inocula are employed. Grows well at 37° C. but not at 22° C. Requires X but not V factor in air containing 5 per cent. CO₂; requires neither factor for anaerobic growth (see below). No growth on MacConkey, glucose or glycerol agar.

Resistance and viability. Two-day broth culture killed in 10 minutes at 60° C. Dies after 4-5 days in broth at 37° C. in air.

Biochemical activities. Ferments glucose and sucrose in 2 days, maltose in 2-3 days, lactose in 3-4 days, starch in 9 days and dextrin and glycogen in 10 days. Does not ferment galactose, fructose, raffinose, xylose, mannitol, dulcitol, sorbitol, salicin or inulin. Litmus milk, slight acidity after 14 days. Does not produce indole; nitrates reduced; M.R. and V.P. negative; methylene blue reductase positive. Tests for NH₃, H₂S and catalase negative.

Pathogenicity. Four guinea-pigs and 8 mice each received intraperitoneally 42×10^4 living organisms without any observable ill effect.

Serology. The suspensions of the various strains isolated varied as regards stability in 0.2 and 0.85 per cent. NaCl. The most stable suspension was that from the 4th blood culture. The 3rd and 4th blood culture strains and the mitral vegetation strain were all agglutinated to the same titre (1:320) by the patient's serum taken on the 60th day of illness, and to full titre by a serum prepared against the 3rd blood culture strain. Macroscopic

agglutination tests at 37° and 55° C. were unsatisfactory owing to the instability of the suspensions. Slide agglutination at 20° C. gave dependable results.

Growth requirements

The growth requirements were tested by seeding graded inocula of thrice-washed bacteria in peptone water suspensions, consisting only of separate cells, into fluid media and agar shakes and on to agar plates. Growth in fluid media was estimated by opacity and confirmed by subculture on Fildes's agar in 5 per cent. CO₂, that on solid media by viable counts as described by Miles and Gray. For V factor 10 per cent. of a yeast extract was added and for X factor a 1 per cent. solution of hæmatin in 1 per cent. Na₂CO₃. The results are shown in tables I and II.

TABLE I

Growth requirements in fluid media

Inoculum (approx.)	Growth in peptone water									Growth in broth	
	Alone			With X factor		With V factor		With X + V factor			
	Air	An.	CO ₂	Air	CO ₂	Air	CO ₂	Air	CO ₂	Air	CO ₂
300,000	+	+	—	—	++	—	—	++	++	+++	+++
30,000	—	+	—	—	++	—	—	++	++	—	+++
3000	—	+	—	—	++	—	—	++	++	—	+++
300	—	+	—	—	++	—	—	++	++	—	+++
30	—	—	—	—	++	—	—	++	+	—	—
3	—	—	—	—	++	—	—	+	+	—	—
0.3	—	—	—	—	—	—	—	—	—	—	—

+, ++, +++ = degrees of growth; An. = anaerobic incubation;
CO₂ = 5 per cent. of CO₂ in air.

In fluid media the organism grows anaerobically without X factor. In this respect it resembles *H. influenzae* (Anderson, 1931). In air both CO₂ and X factor are necessary except in peptone water containing both X and V factors; this CO₂-sparing effect of the yeast extract is surprising and at present inexplicable. The results on solid media (table II) confirm those with fluid media, except that the addition of X and V factors to peptone agar does not permit aerobic growth. This difference may be due to retention in the fluid medium of the CO₂ produced during growth of the organism and its loss at the surface of the solid medium (cf. Wilson, 1931). Studies of growth in agar shakes confirm this supposition. An inoculum of 84,000 organisms failed

to grow in peptone agar and grew feebly in nutrient agar, maximum growth took place in Fildes's agar. If such a shake was incubated in air, growth occurred in the lower two-thirds of the tube, the upper third was clear. Grown in air containing 5 per cent of CO_2 the colonies germinated equally well throughout the culture. Presumably the CO_2 in the aerobic tubes escaped from the agar near the surface. The incorporation of indicator showed that the pH varied between 7.2 and 6.6 according to the penetration of CO_2 . That the stimulating effect of the CO_2 is not due to changes in the pH of the medium is obvious from the following experiment. Plates were prepared from four lots of Fildes's medium buffered to pH 8.0, 7.5, 7.0 and 6.5, respectively. Surface viable counts were made on each lot, 3 plates from each being incubated aerobically and 3 in air + 5 per cent of CO_2 . The most concentrated inoculum contained 2.8×10^6 organisms. This inoculum did not germinate on any of the plates incubated aerobically. On the other hand, the growth in CO_2 was marked in all four lots, the counts (in millions) being at pH 8.0, 2.23, at pH 7.5, 2.10, at pH 7.0, 2.26 and at pH 6.5, 2.83. The colonies were largest on the medium at pH 7.5 (mean diameter 0.66 mm) and smallest at pH 6.5 (0.35 mm). The CO_2 effect is obviously independent of the pH of the medium.

TABLE II

Growth requirements on solid media

Medium	Estimated number of colonies		
	In air	In 5 per cent CO_2 in air	Anaerobically
Peptone agar	30	5	
" + V factor	0	0	
" + X factor	0	2 870 000	
" + X and V factors	0	1,170,000	
Nutrient agar	0	43	625
Blood	70	3 570 000	
Fildes's	70	3,000 000	3,450 000

Inoculum = approximately 3,570 000 viable organisms

It will be seen from table II that on solid media the organism requires CO_2 and X factor but not V factor. Scanty growth occurs aerobically with very heavy inocula. Presumably the thick mass of organisms in such inocula can produce enough CO_2 to initiate growth. The scanty growth on nutrient agar in CO_2 is probably due to a trace of X factor carried over in the inoculum. The dependence of the organism upon Fildes's extract and upon the presence of an adequate concentration of CO_2 in the air is displayed

in table III. There is fair growth in air containing 0.1 per cent. of the gas, but 5 per cent. is the optimum concentration.

TABLE III
Gaseous requirements on solid media

Atmosphere	Number of colonies	
	Fildes's medium	Nutrient agar
Air	2	0
100 per cent. O ₂	0	0
5 per cent. CO ₂ + 95 per cent. O ₂	0	0
CO ₂ in air ca. 0.1 per cent.	3000	0
" " 0.7 "	30,000	0
" " 2.0 "	23,000	0
" " 5.0 "	52,000	0
" " 90.0 "	60	0

Inoculum = 52,000 viable organisms.

Changes in metabolic characteristics with subculture

There were indications in some tests that CO₂ and X factor were, in certain circumstances, not necessary. A few tests indicated that the stock strains were becoming adapted to growth in peptone water alone and the following experiment illustrates an adaptation to growth in the absence of excess of CO₂. Every three days broth subcultures of the strain obtained from the mitral vegetation were made in duplicate and incubated at 37° C., one aerobically, the other in air + 5 per cent. of CO₂. In all, fifty such subcultures were made and on the fiftieth, surface viable counts were made of each "strain" on Fildes's agar plates in air and in air + 5 per cent. CO₂ (Khairat, 1940). The results are shown in table IV.

TABLE IV
Comparison of strains subcultured in air and in air + 5 per cent. of CO₂

Test atmosphere . . .	Subcultured in air + 5 per cent. of CO ₂		Subcultured aerobically	
	Air	Air + CO ₂	Air	Air + CO ₂
Count	0	20 × 10 ⁶	21.5 × 10 ⁶	19.0 × 10 ⁶
Mean colony diameter (mm.)	...	0.40	1.32	1.45

It will be seen that the strain subcultured in air had adapted itself to aerobic cultivation, producing relatively large colonies, but that the strain kept in CO₂ remained incapable of aerobic growth and produced significantly smaller colonies:

CLASSIFICATION OF THE HÆMOPHILIC BACILLUS

The bacillus is non motile, non sporing and Gram negative. It is markedly hæmophilic and resembles *H. canis*, *Bact. influenzae murium* (Ivanovics and Ivanovics, 1937) and Ducrey's bacillus (Lwoff and Pirotsky, 1937) in requiring X but not V factor. The CO₂ requirements and relevant biochemical activities of *H. canis* (strain NCTC 1659) and Ivanovics's bacillus are compared with those of the new strain in tables V and VI. The three strains are

TABLE V

Comparison of the gas requirements of certain hæmophilic bacilli

Atmosphere —	Number of colonies developing from 0.02 c.c. of original culture		Mean diameter of colonies (mm.)	
	Air	5 per cent. of CO ₂ in air	Air	5 per cent. of CO ₂ in air
The new <i>Hæmophilus</i>	0	53 300		1.22
<i>H. canis</i>	2 000 000	2 570 000	1.51	0.66
<i>Bact. influenzae murium</i>	10 000 000	11 700 000	0.98	1.33

TABLE VI

Characters of certain hæmophilic bacilli

	Indole production	Lactose fermentation	Mannite fermentation	CO ₂
The new <i>Hæmophilus</i>	—	A	0	Necessary
<i>H. canis</i>	+	0	A	Not required
<i>Bact. influenzae murium</i>	+	0	0	Not required

A = acid production

distinct and the distinction was confirmed by tests in which a high titre antiserum prepared against the new strain caused no agglutination of the other two. Strains of Ducrey's bacillus were unobtainable and the recorded descriptions of the bacillus are too incomplete for biochemical comparisons. The growth of Ducrey's bacillus is apparently improved by increased CO₂ tension (Hunt, 1935-36) and the reported difficulties in the primary isolation of this organism suggest that its CO₂ requirements may be insufficiently satisfied in the procedures usually employed. Nevertheless, there are no indications in the literature that its CO₂ requirements are as exacting as those of the new strain. The new strain, therefore, may at present be considered as distinct

from Ducrey's bacillus, pending serological and biochemical tests of freshly isolated strains of the latter.

Of the less well defined species included in the *Hæmophilus* group by Bergey (1939), the following are sufficiently well described, and permit the selection of certain features which distinguish them from the new strain. Mackie, van Rooyen and Gilroy's organism (1933) grows well in air without enrichment with X or V factor, ferments mannitol and fails to ferment lactose or sucrose; Martins's *Cocco-bacillus meningitidis* (1928) is motile; Gibbons's new *Hæmophilus* (1929) grows on serum agar, produces NH_3 and ferments fructose, galactose, mannitol, xylose and salicin; Jones and Little's *diplo-bacillus* (1923) is both hæmolytic and proteolytic. The *H. gallinarum* of Eliot and Lewis (1934), though its growth is markedly enhanced in a CO_2 atmosphere, requires V factor, whereas Mitchell's *H. ovis* (1925-26), though requiring X factor, has no special CO_2 requirements and ferments galactose, fructose, xylose, raffinose, mannitol and sorbitol. There is thus no reason for identifying the new strain with any one of these organisms.

The adaptation of the new hæmophilic bacillus to growth in air, the gradual reduction of the stringency of its X factor requirements with prolonged subculture, and the rapid disappearance of the "mulberry" colony form soon after isolation indicate that the organism had certain relatively unstable characteristics. The adaptation to aerobic conditions is not without parallel, for some strains of *Br. abortus* adapt themselves with equal rapidity (A. A. Miles, personal communication). Moreover, the X factor and gas requirements of all the eight freshly isolated strains were so marked and clear-cut as to demand inclusion in the list of definitive taxonomic characteristics of this organism. The organism should, without doubt, be included in the genus *Hæmophilus*. It is suggested tentatively that it should be regarded as a new species. Its striking dependence on CO_2 might well be indicated in the adoption of a specific name embodying its preference for an atmosphere containing an excess of CO_2 . There is no root word (like "carboxy" for CO) signifying CO_2 . By analogy with "anaerobiosis" the term "mephitibiosis" was suggested by Nye and Lamb (1936) and the corresponding adjective "mephitibic" was defined by Dorland (1938) as "Growing under increased carbon dioxide tension: said of certain bacteria". But the Latin *mephitis* suggests foul odours and is more apt for noxious or sulphurous atmospheres than those primarily containing CO_2 ; and the combination of the Latin with the Greek "bios" is indefensible on grounds either of euphony or linguistic consistence. A manifestation of CO_2 well known in classical times was the formation of bubbles of gas in fermenting wine; i.e. the froth, or "aphros." Moreover there is in "aphros" a direct connection with

the carbon dioxide of the early days of modern chemistry, for van Helmont collected his *gas sylvestre* from the wine vat. The specific name *aphrophilus* is suggested for the organism.

SUMMARY AND CONCLUSIONS

A hæmophilic organism is described as the cause of a fatal case of bacterial endocarditis. It differs in certain respects from all well established species in the genus *Hæmophilus*, though its X and V factor requirements resemble those of *H. canis*. It shares with *Brucella abortus* the property of requiring relatively high concentrations of CO₂ for isolation on the usual media. It does not correspond with any recorded description of hæmophilic bacilli and it is tentatively put forward as a new species, with the name *H. aphrophilus*. The occurrence of a case of endocarditis due to a hæmophilic bacillus which, on investigation, proves to be other than *H. influenzae* strengthens the suggestion made by Miles and Gray that the true influenza bacillus is only rarely the cause of human endocarditis.

My thanks are due to Professor G. R. Cameron for the post mortem findings, to Dr Kenneth Harris for permission to publish the clinical history, to Dr G. Ivánovics for the strain of *Bact. influenzae murum*, to Mr A. P. Sinker for his help in naming the organism and to Professor A. A. Miles for his interest and advice.

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CHEMOTHERAPY OF THE EXPERIMENTAL TYPHOID AND PARATYPHOID CARRIER CONDITION IN THE IMMATURE RABBIT

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THE investigations previously described with reference to experimental infection in immature (Dutch) rabbits with *B. typhosus* (Coplans, 1935, 1936) have been extended and again confirmed after an interval of two years. Two parallel series of investigations have also been made with *B. paratyphosus* A and B.

In each animal, infection has been procured by means of a single intravenous dose of the suspension of organisms in a quantity of normal saline equivalent to 0.5 c.c. per kg. The initiation of experimental therapeutic measures has been limited to the period between the 30th day preceding and the 60th day following inoculation. Premedication, when practised, has always been carried out up to the day of inoculation and followed by systematic medication commencing within 24 hours. The treatment, both premedication and medication, has been limited to oral administration once daily of hexamine sodium acetate (0.2 g. hexamine and 0.4 g. sodium acetate) dissolved in about 1.25 c.c. of distilled water. This dosage has been given irrespective of age or weight.

The systematic post mortem examination for the presence of the specific infecting organism has included, in all animals, heart blood, spleen, liver, gall-bladder, bile, duodenal contents, kidney, urinary bladder, urine, faeces (rectal contents), and bone marrow, in those animals in which infection has been attempted by repeated oral administration of suspensions of the pathogenic organisms the stomach contents were also examined. The method of examination of the tissues and excreta and the special nutrient media employed have already been described (Coplans, 1935, 1936).

EXPERIMENTS WITH *B. TYPHOSUS*

Series T 10, 11, table I (fifth animal passage), May and July 1936.
In all 58 animals were inoculated and examined, 19 untreated and 39 treated. The control animals were kept under observation for varying periods extending to 79 days after inoculation. Medication was delayed until the 60th day after inoculation, and it was found that the animals became entirely free of the infecting organism after 17 days' treatment. The long delay in the commencement of treatment has therefore been without appreciable deleterious effect.

Premedication was commenced 30, 21 and 15 days respectively prior to inoculation and was followed by medication daily. With 30 days' premedication the animals were found to be entirely free of the infecting organism 6 days after inoculation. With 21 or 15 days' premedication they were free 7 days after inoculation. Thus no important advantage appears to be gained by commencing premedication at any period earlier than 10 days before actual infection with *B. typhosus*.

TABLE I

Schedule of experiments with B. typhosus in immature rabbits

Series	Experimental procedure (dose 0.2 agar slope per kg.)	Number of animals used	Limit of experiment (days)	Treatment		
				begun		ended
				before inoculation (premedica- tion) (days)	after inoculation (medication) (day-)	after inoculation (days)
T. 10	1. Inoculated: untreated	6	79
	2. " treated	5	79	...	60	77
T. 11	1. " untreated	13	21
	2. " treated	11	30	15	...	9
	3. " "	11	39	21	...	9
	4. " "	12	44	30	...	9
T. 12	1. " untreated	7	44
	2. " treated	10	28	...	3	20
T. 13, T. 14	" untreated	7	69
T. 15	" untreated	3	47
T. 16	1. " untreated	8*	21
	2. " treated	8	27	10	...	10
Special	" untreated	5	2

* One early death: examination not recorded.

Series T 12-16, table I (tenth animal passage). April to August 1938. The pathogenic strain, after propagation by monthly subcultures on sloped nutrient agar for 2 years, was again passed in succession through 5 immature rabbits by intravenous inoculation, the infecting organism being recovered from the blood in each inoculated animal 24-48 hours later. In each passage the presence of *B. typhosus* in the bile was verified. In all, 43 animals were inoculated and examined, 25 untreated and 18 treated. The limit of observation of untreated animals was 69 days following inoculation. Treatment was as follows: (1) premedication for 10 days before inoculation, with treatment continued for a further 10 days; (2) medication commencing 3 days after inoculation

and continued for a further 17 days. The results show that with premedication the animals became free of infection 7 days after inoculation, and in those animals in which treatment was begun after infection had taken place, freedom from infection was obtained after 17 days' treatment. These results confirm those obtained 2 years earlier with animals in which infection constituted the 5th animal passage.

Onset of infection. (Special series, table I). Following a single intravenous injection the organism was recovered from the body, as follows.

After 3 hours	heart blood, spleen, liver, bile and kidney.
After 9, 18, 30 hours	gall-bladder, duodenal contents and urinary bladder.
After 48 hours	fæces, bone-marrow and urine.

In untreated animals the distribution and persistence of the infecting organisms generally throughout the body were found to resemble those previously described for fifth passage inoculations.

EXPERIMENTS WITH *B. PARATYPHOSUS* A AND B

The strains used in these experiments—*B. paratyphosus* A "Mears" and *B. paratyphosus* B "Rowlands"—were obtained from the Royal Army Medical College on 1st October 1936. The former was originally isolated in November 1915 from the blood of a case of paratyphoid from Gallipoli. These organisms had not previously been passed through the rabbit.

The method of preparation of the organisms for these experiments, *i.e.* adaptation to infection of young rabbits by passage, was similar to that followed with *B. typhosus*. Each of the strains was passed by intravenous inoculation through a series of immature animals in succession and isolated from the blood after each passage, an 18-hour subculture on an agar slope at 37° C. providing the suspension for the succeeding passage. The quantity of organisms injected varied from 0.25 agar slope per kg. body weight in the initial passage to a minimum of 0.005 in the fifth passage, quantities less than this failing to infect.

For purposes of comparison a fifth passage was also made upon a series of animals with a culture of each organism derived from the infected bile of a rabbit of the fourth passage. The minimal infecting dose was found to be 0.05 agar slope per kg. of *B. paratyphosus* A and half this quantity of *B. paratyphosus* B. Thus a single passage of either of these organisms through the bile results in their virulence being markedly reduced (table II).

TABLE II

Animal passage experiments with B. paratyphosus A and B

Rabbit passage	Source of culture	Weight of rabbit (g.)	Dosage (agar slope per kg.)	Result	Bacteriological examination										
					Blood	Spleen	Liver	Gall-bladder	Bile	Duodenal contents	Kidney	Urinary bladder	Urine	Faeces	Bone-marrow
B. paratyphosus A (Mears)															
1st	Stock agar slope	670	0.25	K. 96th hour	+	+	-	+	+	+	+	-	-	+	-
2nd	Blood rabbit, 1st passage	555	0.5	" 46th "	+	+	+	+	+	+	+	+	+	+	+
3rd	" " 2nd "	745	0.4	D. 74th "	+	+	+	+	+	+	+	+	+	+	+
4th	" " 3rd "	750	0.1	K. 10th day	+	-	+	+	+	+	+	+	+	+	+
	" " " "	580	0.05	" " "	+	+	+	+	+	+	+	+	+	+	+
5th	" " 4th "	505	0.1	D. 4th "	+	-	+	+	+	+	+	+	+	+	+
	" " " "	690	0.05	K. 8th "	+	-	+	+	+	+	+	+	+	+	+
	" " " "	730	0.025	D. 7th "	+	-	+	+	+	+	+	+	+	+	+
	" " " "	490	0.0125	K. 11th "	+	-	+	+	+	+	-	+	+	+	+
	" " " "	520	0.005	" " "	+	-	+	+	+	+	-	+	+	+	+
	" " " "	480	0.0025	" " "	-	-	-	-	-	-	-	-	-	-	-
5th	Bile " " "	700	0.05	" 13th "	+	-	+	+	+	-	+	+	+	+	+
	" " " "	480	0.025	" 11th "	-	-	-	-	-	-	-	-	-	-	-
	" " " "	420	0.0125	" " "	-	-	-	-	-	-	-	-	-	-	-
	" " " "	510	0.005	" " "	-	-	-	-	-	-	-	-	-	-	-
	" " " "	505	0.0025	" " "	-	-	-	-	-	-	-	-	-	-	-
B. paratyphosus B (Rowlands)															
1st	Stock agar slope	580	0.25	D. 90th hour	+	+	+	+	+	+	+	+	+	+	+
2nd	Blood rabbit, 1st passage	650	0.25	" 46th "	+	+	+	+	+	+	+	+	+	+	+
3rd	" " 2nd "	775	0.2	" 72nd "	+	+	+	+	+	+	+	+	+	+	+
4th	" " 3rd "	760	0.05	K. 10th day	+	+	+	+	+	+	+	+	+	+	+
	" " " "	585	0.025	" 10th "	+	+	+	+	+	+	+	+	+	+	+
5th	" " 4th "	400	0.05	D. 2nd "	+	+	+	+	+	+	+	+	+	+	+
	" " " "	665	0.025	" 3rd "	+	+	+	+	+	+	+	+	+	+	+
	" " " "	470	0.0125	K. 11th "	+	-	+	+	+	+	+	+	+	+	-
	" " " "	450	0.005	" " "	+	-	+	+	+	+	+	+	+	+	-
	" " " "	490	0.0025	" " "	-	-	-	-	-	-	-	-	-	-	-
5th	Bile " " "	680	0.05	" 13th "	+	+	+	+	+	+	+	+	+	+	+
	" " " "	505	0.025	" " "	+	+	+	+	+	+	+	+	+	+	+
	" " " "	460	0.0125	" 11th "	-	-	-	-	-	-	-	-	-	-	-
	" " " "	450	0.005	" " "	-	-	-	-	-	-	-	-	-	-	-
	" " " "	490	0.0025	" " "	-	-	-	-	-	-	-	-	-	-	-

+ = organism present ; - = organism not found. K. = killed ; D. = died.

The experiments summarised in tables III and V constitute a fifth animal passage.

TABLE III

Schedule of experiments with *B. paratyphosus A* in immature rabbits

Series	Dose (agar slope per kg)	Experimental procedure	Number of animals used	Early deaths (examinations not recorded)	Limit of experiment (days)	Treatment		
						begun		ended
						before inoculation (premedication) (days)	after inoculation (medication) (days)	
A. 1	0.1	1. Inoculated: untreated 2. "	5 5	0 0	367 60 3	... 47
A. 2	0.1	1. " untreated 2. " treated	28 12	13 0	477 68 3	... 40
A. 3	0.1	" untreated	34	26	386
A. 4	0.05 0.025	1. " untreated 2. " "	3 3	0 0	8 8
A. 5	0.025	1. " untreated 2. " treated	24 6	0 0	359 84 30	... 70
A. 6	0.02	1. " untreated 2. " treated	46 10	7 0	344 97 30	... 70
A. 8	0.02	1. " untreated 2. " treated 3. " " 4. " " 5. " "	11 20 9 9 9	0 0 0 0 0	81 42 47 52 57 5 10 15	... 3 34 27 27 25
A. 9	0.5	Mouth feeding daily: untreated	10	0	37
A. 10	0.02	1. Inoculated: untreated 2. " treated	8 15	0 0	85 12 3	... 38
Special	0.02	" untreated	6	0	1

Animal experiments with *B. paratyphosus* A

Onset of infection. (Special series, table III). Following the intravenous injection of a single dose of 0.02 agar slope per kg. —four times the minimal infecting quantity—the infecting organism was recovered from the body as follows.

After 2-3 hours . . .	heart blood, spleen, liver and kidney.
„ 3½ „ . . .	urinary bladder.
„ 9 „ . . .	gall-bladder and bile.
„ 18 „ . . .	duodenal contents.
„ 24 „ . . .	faeces, bone-marrow and urine.

Distribution and persistence of infection. In the 107 surviving untreated control animals which proved positive when examined between the 1st and 53rd week following inoculation with doses varying from 0.02 to 0.1 agar slope per kg. the infecting organism was recovered as shown in table IV.

TABLE IV

Recovery of B. paratyphosus A from untreated rabbits

Tissue, etc.	Period (weeks)	No. examined	No. positive	Percentage positive	Last day positive
Blood	1-4	72	36	50.0	28th
	5-6	5	1	20.0	31st
	7-13	21	1	4.8	65th
	14-53	9	0	0	...
Spleen	1-4	72	30	41.7	27th
	5-6	5	0	0	...
	7-13	21	2	9.5	67th
	14-53	9	0	0	...
Bone-marrow	1-4	72	58	80.6	27th
	5-6	5	4	80.0	42nd
	7-13	21	12	57.1	85th
	14-53	9	0	0	...
Liver	1-53	107	100	93.4	367th
Gall-bladder	1-53	107	106	99.2	367th
Bile	1-53	107	101	97.2	253rd
Duodenal contents	1-13	98	78	79.6	85th
	14-53	9	1	11.1	142nd
Kidney	1-2	52	30	57.6	14th
	3-4	20	1	5.0	23rd
	5-6	5	1	20.0	30th
	7-13	21	0	0	...
	14-53	9	0	0	...
Urinary bladder	1-13	98	81	82.6	85th
	14-53	9	0	0	...
Urine	1-13	98	81	82.6	85th
	14-53	9	0	0	...
Faeces	1-13	98	71	72.4	90th
	14-53	9	0	0	...

B. paratyphosus A in the excreta. Of the 98 animals examined during the first 13 weeks the urine was found positive in 81 and the faeces in 71, the distribution being as follows.

Faeces +, urine +	.	.	59 animals (60.2 per cent.).
" +, " -	.	.	12 " (12.2 ").
" -, " +	.	.	22 " (22.4 ").
" -, " -	.	.	5 " (5.1 ").

Of the animals thus examined 93 (94.9 per cent.) were found to be excreting the organism in faeces or urine or both, while 5 (5.1 per cent.) proved negative in both.

Hepatic system. Infection of the liver and gall-bladder proved to be the longest standing of all the tissue infections examined, persisting into the 53rd week following inoculation, whereas the bile was found to be infective up to the 37th week only. The organism was recovered from the duodenal contents up to the 21st week and from the faeces up to the 13th week only.

Infection of the urinary tract. The following is the record of the 98 animals examined at the same time as to the condition of the kidney, the urinary bladder and the urine during the first 13 weeks following infection.

Kidney +, urinary bladder +, urine +	.	.	31 animals (31.6%)
" -, " " +, " +	.	.	50 " (51.0%)
" +, " " -, " -	.	.	1 animal (1.0%)
" -, " " -, " -	.	.	16 animals (16.3%)

In 81 (82.6 per cent.) of these animals the organism was recovered from both bladder and urine, and in 31 of these there was also infection of the kidney. There was only one instance of infection of the kidney alone; there was no case of infection of the urinary bladder alone or of the urine alone.

*Chemotherapy: treatment with hexamine-sodium acetate **

Medication only. Treatment commenced 3rd day after inoculation. (Series A. 1, 2, 8 and 10, table III.) In 52 animals so treated the tissues and excreta were found to become free of infection at the following times.

After 5 days' treatment	.	kidney, urinary bladder, urine and faeces.
" 9 "	.	blood.
" 16 "	.	spleen.
" 29 "	.	bone-marrow and duodenal contents.
" 30 "	.	bile.
" 32 "	.	liver.
" 34 "	.	gall-bladder.

* The detailed protocols of these and other experiments recorded in this paper have been deposited with the Librarian, General Library, British Museum (Natural History), London, S.W. 7.

One animal of the series was found to be entirely free of infection after 30 and two after 32 days' treatment and the remaining 15 animals after treatment lasting 34 days or more.

Treatment commenced 30 days after inoculation. (Series A. 5 and 6, table III.) Sixteen animals received medication daily from the 30th day following inoculation. In these the blood was found negative after the first day's treatment; the urinary bladder, urine and faeces were regularly free of infection after 5 days, the bone-marrow and spleen after 8 days, the duodenal contents after 31 days, the bile and gall-bladder after 36 days and the liver after 37 days. The 8 surviving animals treated 37 days or longer proved entirely negative.

Premedication followed by medication. (Series A. 8, table III.) Twenty-seven animals were so treated, 9 receiving premedication for 5 days, 9 for 10 days and 9 for 15 days before inoculation, and the earliest examination of these animals was made 20, 12 and 8 days respectively after inoculation.

With premedication lasting 15 days the result of examination was as follows. In no animal was the infecting organism recovered from the spleen, urinary bladder, urine or faeces at the first examination (8th day) or later. The bone-marrow proved negative on the 14th day, the bile on the 21st day, and the liver, gall-bladder and duodenal contents on the 23rd day after inoculation. The 4 animals which were examined on the 23rd day or subsequently proved entirely negative.

With premedication limited to 10 and 5 days respectively similar results were obtained, but in the 13 animals treated 24 days or longer after inoculation, the results were entirely negative.

The general advantage of this form of treatment as compared with medication alone commencing on the third day after inoculation is that with the former entire freedom from infection is obtainable after 24 days' treatment subsequent to inoculation, whereas, with the latter, freedom appears to be established only after 32 days' treatment or longer, *i.e.* not less than 35 days after inoculation. When the commencement of treatment (medication only) is still further delayed to the 30th day subsequent to inoculation, freedom from infection appears to be established only after 37 days' treatment, *i.e.* 67 days after inoculation.

Animal experiments with *B. paratyphosus* B

Onset of infection. (Special series, table V.) Following intravenous injection of a single dose only of 0.025 agar slope per kg.

TABLE V
Schedule of experiments with *B. paratyphosus* B in immature rabbits

Series	Dose (agar slope per kg)	Experimental procedure	Number of animals used	Early deaths (examinations not recorded)	Limit of experiment (days)	Treatment		
						before inoculation (premedication) (days)	began after inoculation (medication) (days)	ended after inoculation (days)
B. 1	0.05	Inoculated; untreated	13	3	313
B. 2	0.025	1. untreated	19	1	38
		2. treated	13	0	39	...	3	17
B. 3	0.025	1. untreated	16	5	120
		2. treated	8	0	91	...	3	11
B. 4	0.025	1. untreated	21	1	369
		2. treated	11	0	121	...	30	77
B. 5	0.025	1. untreated	12	0	333
		2. treated	5	0	52	5	...	32
		3. "	5	0	48	10	...	31
		4. "	7	0	46	15	...	31
		5. "	6	0	74	...	30	74
B. 6	0.5	Mouth feeding daily, untreated	10	0	37
B. 7	0.025	1. Inoculated; untreated	10	0	16
		2. treated	10	0	41	...	3	11
Special	0.025	" untreated	4	0	1
Mixed infection with <i>B. paratyphosus</i> B and <i>B. typhosus</i> .								
{ B. 7 and " T. 12 { <i>B. typhosus</i>	{ 0.025 0.2	1. Inoculated; untreated	12	0	44
		2. " treated	13	0	42	...	3	42

(five times the minimal infecting quantity), the infecting organism was recovered from the body as follows.

After 3-9 hours	.	.	heart blood, spleen, liver, gall-bladder, bile and kidney.
„ 18	„	.	duodenal contents, urinary bladder, urine and bone-marrow.
„ 24	„	.	fæces.

Distribution and persistence of infection. In the 78 surviving untreated animals which proved positive when examined between the 1st and 47th week following inoculation (two of this number being examined as to bile only, following laparotomy), the dosage varied from 0.025 to 0.05 agar slope per kg. and the infection was distributed as shown in table VI.

B. paratyphosus B in the excreta. As shown, positive results were obtained on examination of the fæces in 51 and in the urine in 67 of the 69 animals examined up to the 91st day after inoculation. These were distributed in the following way.

Fæces +, urine +	.	.	49 animals (71 per cent.).
„ +, „ -	.	.	2 „ (3 „).
„ -, „ +	.	.	18 „ (26 „).
„ -, „ -	.	.	nil.

All the animals examined were found to be excreting the pathogenic organism in the fæces or urine or both.

Hepatic system. This proved to be the nidus of infection of longest persistency, the liver substance being positive up to the 35th week, the bile up to the 41st week, and the gall-bladder up to the 47th week after infection. The infecting organism was recovered from the duodenal contents up to the 35th week, but from the fæces only up to the 10th week after inoculation.

Infection of the urinary tract. The following is the record of the 69 animals examined *post mortem* as to the condition of the kidney, urinary bladder and urine during the first 13 weeks following infection.

Kidney +, urinary bladder +, urine +	.	.	31 animals (44.9%)
„ -, „ -, „ +	.	.	33 „ (47.8%)
„ +, „ -, „ +	.	.	1 „ (1.4%)
„ -, „ -, „ +	.	.	2 „ (2.9%)
„ -, „ -, „ -	.	.	2 „ (2.9%)

In 64 animals (92.7 per cent.) the infecting organism was recovered from both urinary bladder and urine, and in 31 of these

there was an associated infection of the kidney. In the whole of the 69 animals thus examined there are only two instances in which the urine alone was found infected. In no animal was the kidney alone or the urinary bladder alone found infected. In one animal both kidney and urine were found infected but not the urinary bladder. In two animals the kidney, urinary bladder and urine all proved negative.

TABLE VI

Recovery of B paratyphosus B from tissues and excreta

Tissue etc	Period (weeks)	No examined	No positive	Percentage positive	Last day positive
Blood	1-4	50	22	44.0	27th
	5-6	11	4	36.3	37th
	7-13	8	0	0	
	14-47	7	2	28.6	112th and 320th respectively
Spleen	1-4	50	31	62.0	27th
	5-6	11	3	27.3	37th
	7-13	8	2	25.0	91st
	14-47	7	1	14.0	112th
Bone marrow	1-4	50	35	70.0	27th
	5-6	11	10	90.9	41st
	7-13	8	3	37.5	69th
	14-47	7	1	14.0	112th
Liver	1-47	76	72	94.6	242nd
Gall bladder	1-47	76	75	98.6	320th
Bile	1-47	76	74	97.3	242nd
" (laparotomy)		1	1		261st
"		1	1		282nd
" (total)	1-47	78	76	97.4	282nd
Duodenal contents	1-13	69	47	68.1	80th
	14-47	7	2	28.6	242nd
Kidney	1-2	38	31	82.1	13th
	3-4	12	1	8.3	27th
	5-6	11	0	0	
	7-13	8			
	14-47	7			
Urinary bladder	1-13	69	64	92.7	80th
	14-47	7	0	0	
Urine	1-13	69	67	97.1	91st
	14-47	7	0	0	
Feces	1-13	69	51	73.9	64th
	14-47	7	0	0	

Chemotherapy: treatment with hexamine-sodium acetate

Medication only. *Treatment commenced 3rd day after inoculation.* (Series B. 2, 3 and 7, table V.) In 31 animals so treated freedom from infection was demonstrated at the following times.

After 5 days' treatment	.	blood, kidney, urinary bladder, urine and fæces.
" 15 "	"	spleen.
" 25 "	"	liver and duodenal contents.
" 32 "	"	bile.
" 36 "	"	bone-marrow.
" 37 "	"	gall-bladder.

Seven animals receiving treatment for 37 days or more proved entirely negative. One rabbit of series B. 3, killed after 35 days' treatment, proved to be positive in the bone-marrow and negative in all remaining tissues and excreta examined.

Treatment begun 30 days after inoculation. (Series B. 4 and 5, table V.) Seventeen animals treated from the 30th day following inoculation received medication daily. The spleen, urinary bladder, urine and fæces were negative after 5 days, the bone-marrow after 16 days, the blood, bile and duodenal contents after 24 days and the liver and gall-bladder after 43 days of treatment. The 7 surviving animals receiving treatment for 43 days or more proved entirely negative.

Premedication followed by medication. (Series B. 5, table V.) Five animals received premedication for 5 days before inoculation, 5 for 10 days and 5 for 15 days, and the earliest examination of an animal of each group was made on the 5th day following inoculation. In no animal which received 10 or 15 days' premedication was the pathogenic organism recovered from the blood, spleen, urinary bladder, urine, fæces or bone-marrow on the 5th day after inoculation or later. The duodenal contents proved negative on the 15th day, the liver and bile on the 27th day and the gall-bladder on the 29th day following inoculation. The 4 animals which were examined on the 29th day or subsequently proved entirely negative.

With premedication limited to 5 days only, the results are somewhat similar, but entirely negative results were obtained in 3 animals examined on the 31st day following inoculation, or later.

The results show the general advantage of commencing treatment as early as possible in order to free the inoculated animal of all infection. With premedication for 10 or 15 days followed by medication, entire freedom from infection is found after 29 days' treatment subsequent to inoculation; and with premedication limited to 5 days only, after 31 days of post-inoculation treatment.

With treatment (medication alone) commencing 3 days after inoculation, freedom from infection appears established after 37 days' treatment or longer, *i.e.* not less than 40 days after inoculation. When the commencement of treatment (medication only) is delayed to the 30th day after inoculation freedom from infection appears to be established only after 43 days' treatment, *i.e.* 73 days after inoculation.

MIXED INFECTION WITH *B. TYPHOSUS* AND *B. PARATYPHOSUS* B.
(TABLE V, SERIES B. 7, T. 12)

Twenty-five immature animals were inoculated, 12 untreated and 13 treated, each receiving a single intravenous inoculation of a mixture of saline suspensions of *B. typhosus* (0.2 agar slope per kg.) and *B. paratyphosus* B (0.025 agar slope per kg.). The *B. typhosus* suspension alone was also administered intravenously in similar dosage to 17 immature animals (series T. 12, table I)—7 untreated and 10 treated—and the *B. paratyphosus* B suspension alone similarly to 20 animals (series B. 7, table V)—10 untreated and 10 treated. All the 62 animals of the three series (T. 12; B. 7, T. 12; B. 7) were inoculated on the same occasion. Treatment with hexamine and sodium acetate was begun on the 3rd day after inoculation and continued at 24-hour intervals. Both treated and untreated animals were examined from the 4th to the 44th day after inoculation. The following is the order in which the treated animals became entirely free from infection.

<i>B. typhosus</i> (series T. 12)	after 17 days' treatment.
<i>B. paratyphosus</i> B (series B. 7)	„ 37 „ „
<i>B. typhosus</i> and <i>B. paratyphosus</i> B, mixed. (Series B. 7, T. 12)	„ 39 „ „

The distribution and persistence of the infecting organisms in the animals with the mixed infection closely resembled those in the animals infected with *B. paratyphosus* B alone.

FEEDING EXPERIMENTS WITH *B. PARATYPHOSUS* A AND B

Two groups of ten animals (series A. 9, table III, and B. 6, table V) were fed daily at 24-hour intervals for 30 days with suspensions of these organisms in normal saline, the dose, irrespective of the weight of the animal, being 0.5 agar slope per animal of an 18-hour culture at 37° C. The organisms administered during the first 15 days were isolated from the 5th rabbit passage blood and during the last 15 days from the bile of an animal inoculated with organisms derived from the 4th rabbit passage blood. The animals were examined *post mortem* from the 4th to the 37th day after the commencement of feeding. They were killed for examination at

intervals of 24 hours or more after the last dose. The results of bacteriological examination are recorded in table VII.

TABLE VII

Recovery of organisms from rabbits infected by feeding

Source	Total doses (each of 0.5 agar slope)	Weight		Survived (days)	Bacteriological examination											
		Initial (g.)	Post mortem (g.)		Gastric contents	Blood	Spleen	Liver	Gall-bladder	Bile	Duodenal contents	Kidney	Urinary bladder	Urine	Faeces (rectal)	Bone-marrow
<i>B. paratyphosus A</i>																
Blood	4	620	625	4 (D.)	+	-	-	-	-	-	+	-	-	-	-	-
	7	535	500	7 (D.)	-	-	-	-	-	-	-	-	-	-	+	-
	7	625	600	7 (K.)	-	-	-	-	-	-	+	-	-	-	-	-
	11	680	540	11 (D.)	+	-	-	-	-	-	+	-	-	-	+	-
	11	710	640	11 (D.)	-	-	-	-	-	-	-	-	-	-	+	-
Bile	21	520	530	21 (K.)	+	-	-	-	-	-	+	-	-	-	+	-
	23	710	700	23 (K.)	-	-	-	-	-	-	+	-	-	-	-	-
	30	685	730	33 (K.)	+	-	-	-	-	-	-	-	-	-	+	-
	30	540	515	35 (D.)	-	-	-	-	-	-	-	-	-	-	-	-
	30	710	745	37 (K.)	-	-	-	-	-	-	-	-	-	-	-	-
<i>B. paratyphosus B</i>																
Blood	4	490	500	4 (D.)	+	-	-	-	-	-	+	-	-	-	-	-
	7	580	575	7 (K.)	+	-	-	-	-	-	-	-	-	-	+	-
	11	520	500	11 (D.)	+	-	-	-	-	-	-	-	-	-	-	-
	14	575	550	14 (D.)	+	-	-	-	-	-	-	-	-	-	-	-
Bile	21	540	575	21 (K.)	-	-	-	-	-	-	+	-	-	-	-	-
	23	620	625	23 (K.)	-	-	-	-	-	-	-	-	-	-	+	-
	30	610	610	33 (K.)	-	-	-	-	-	-	-	-	-	-	+	-
	30	570	575	37 (K.)	-	-	-	-	-	-	-	-	-	-	-	-
	30	605	650	37 (K.)	-	-	-	-	-	-	-	-	-	-	-	-
	30	545	555	37 (K.)	-	-	-	-	-	-	-	-	-	-	-	-

D. = died ; K. = killed.

In the paratyphoid A group the dose given by the mouth daily—0.5 agar slope—contained approximately 157 minimal infecting intravenous doses in the case of the blood-derived culture and 15.7 such doses of the culture derived from the bile. The organism was recovered from the alimentary canal in each of the 8 animals examined from the 4th to the 33rd day, but not later—gastric contents 4, duodenal contents 5, faeces 5 animals. In the animal which was killed on the 33rd day, i.e. 72 hours after the last dose, the organism was proved to be present in both gastric contents and faeces.

In the paratyphoid B group the daily dose contained approximately 176 minimal infecting intravenous doses of the blood-derived

cultures and 35.3 such doses of the cultures derived from the bile. The organism was recovered from the alimentary canal in each of the 7 animals examined from the 4th to the 33rd day, but not later—gastric contents 4, duodenal contents 2, faeces 3 animals. In an animal killed on the 7th day the organism was recovered from the gastric contents 24 hours after the last oral administration took place, and in one killed on the 33rd day, *i.e.* 72 hours after the last oral administration, it was recovered from the faeces.

In neither group was the organism recovered from any internal organ or tissue of an animal under test and there was no evidence of systemic infection. There is here ample evidence, however, that in the case of young rabbits, these organisms when ingested by the mouth may be excreted by the bowel unchanged 72 hours later.

EFFECT OF SIZE OF DOSE ON THE DURATION OF INFECTION

The minimal infecting (intravenous) dose for immature rabbits of the strains of *B. paratyphosus* A and *B. paratyphosus* B used in these experiments has been shown to be of the order of 0.005 agar slope per kg. for each organism, the result of inoculation being a typical generalised infection of the tissues, bile, etc., with excretion of the organisms in the urine and faeces (5th rabbit passage, table II). Doses smaller than this failed to produce any observable infection, the result of examination for the presence of the pathogenic organism in the body and excreta proving entirely negative after a few days only. Similar phenomena were observed when inoculation was performed with organisms isolated from the bile of infected animals—after 4 rabbit blood passages, the minimal infecting dose for *B. paratyphosus* A was 0.05 and for *B. paratyphosus* B 0.025 agar slope per kg. The evidence that the inoculated animal completely destroys a subminimal infecting dose leads to the presumption that there exists a certain ascertainable degree of immunity or protective mechanism natural to the immature animal capable of direct measurement by this means. The result of inoculation, within a few days, is a clear-cut picture one way or the other; it is a case of "all or nothing," either the typical generalised infection or its complete absence.

The doses employed for these experiments have considerably exceeded the minimal infecting dose. In the case of *B. paratyphosus* A the single intravenous inoculation given to each animal was 5-20 times and with *B. paratyphosus* B 4-10 times this quantity, and there has resulted a dependable, considerable and sufficiently lasting degree of infection, as shown by the untreated controls, for the carrying out of experimental therapy under controlled conditions.

B. paratyphosus A infection. (a) Animals receiving 0.1 agar slope per kg. (20 minimal infecting doses). (Series A. 1, 2 and 3, table III.) In all, 84 were inoculated of which 39 (46.4 per cent.) died unexamined within 48 hours. Of the 45 survivors 17 received treatment as described and 28 remained under observation untreated. Of the latter, 9, 5 and 6 died in the 1st, 2nd and 3rd weeks respectively, leaving only 8, of which 2 died and 1 was killed in the period 5-12 weeks after inoculation. There were thus left only 5 animals for further observation during the period 13 weeks and later. Of these one died in the 19th week, one was killed in the 39th week and another in the 53rd week. Up to this time all the untreated controls had proved positive on examination. The two surviving animals examined in the 56th and 69th weeks after inoculation proved negative.

(b) Animals receiving 0.05 agar slope per kg. (10 minimal infecting doses). (Series A. 4.) Three animals were inoculated and were killed 8 days later. All proved positive.

(c) Animals receiving 4 or 5 minimal infecting doses (0.02 or 0.025 agar slope per kg.). (Series A. 4, 6, 8 and 10.) In all 170 were inoculated, of which 7 (4.1 per cent.) died unexamined within 48 hours. Of the 163 survivors 78 received various forms of treatment as described and 85 remained under observation untreated. Of the latter, 47 died and 2 were killed during the period 1-4 weeks, and 14 died and 7 were killed during the period 5-12 weeks following inoculation, thus leaving 15 survivors for further observation from the 13th week onwards. Of these, 3 died (13th, 21st and 40th weeks) and 5 were killed (13th, 14th (2 animals), 23rd and 49th weeks). All untreated animals examined from the 48th hour onwards proved positive up to the 49th week. The 7 survivors were killed from the 50th to the 52nd week after inoculation and all proved entirely negative on examination.

Against the slightly longer duration of infection (4 weeks), seen only in a single animal examined in the 53rd week which had received the highest inoculation dose, there is the outstanding disadvantage that with the heavy inoculating dose there follows within 48 hours a mortality of 46.4 per cent. as compared with 4.1 per cent. among the animals receiving the light dose.

B. paratyphosus B infection. (a) Animals receiving 0.05 agar slope per kg. (10 minimal infecting doses). (Series B. 1, table V.) Eleven animals were inoculated, of which 3 died unexamined within 48 hours; of the 8 survivors 7 died by the 9th day, all proving positive. The last survivor of the group died in the 45th week after inoculation and on examination proved negative.

(b) Animals receiving 0.025 agar slope per kg. (5 minimal infecting doses). (Series B. 2-5 and 7, table V.) Of 146 inoculated 7 died unexamined within 48 hours. Of the 139 survivors 65

received various forms of treatment as described, while the remaining 74 were kept under observation untreated. All the animals which were examined at intervals up to the 47th week after inoculation, proved positive. The 3 surviving animals killed subsequently all proved negative.

The heavier infecting dose resulted in a higher mortality among the inoculated animals as compared with the lighter and, moreover, there is no evidence that the heavier dosage produced a longer duration of infection, even in a single animal.

On the whole, increasing the inoculum beyond the limit of 4 or 5 times the minimal infecting dose cannot be shown to have resulted in any particular advantage with either organism in young animals.

DISCUSSION

The passage of selected strains of the enteric group of organisms—*B. typhosus* and *B. paratyphosus* A and B—through a succession of immature rabbits aged about 3 months by means of intravenous inoculation and subsequent isolation from the blood has resulted in the adaptation of these organisms so that with the fifth and subsequent passages in these young animals there is set up a chronic "carrier" condition, the observed limit of infection being, respectively, 253, 367 and 325 days (table VIII, A). It has been found that a measurable minimal infecting dose or quantity of such organisms is required to set up a general infection, whereas a subminimal dose is entirely without effect; there is thus also presented a method of estimating the capacity of the animal's natural resistance to infection by *B. paratyphosus* A and B.

In the case of *B. paratyphosus* A a single passage of the organism through the bile of the inoculated rabbit after a succession of blood passages is sufficient to necessitate a tenfold increase in the minimum quantity of organisms required to produce the usual infection following intravenous inoculation: in short, the immediate reduction in the virulence of the organism following a single bile passage is 90 per cent. Similarly with *B. paratyphosus* B, following a single bile passage a fivefold increase is required, the reduction in virulence amounting to 80 per cent.

With organisms derived from the fifth blood passage, infection of the hepatic system, in particular the gall-bladder and bile, is generally of the longest duration, but there are exceptions in which it is found that the bile is no longer infected and the organism may still be found circulating in the blood stream, a condition somewhat analogous to that found in the human "precocious" typhoid carrier; or the bone-marrow is found infected, especially in animals undergoing treatment by chemotherapy, when the organism can no longer be found in other parts of the body.

controllable by the oral administration of hexamine combined with sodium acetate, the beneficial cumulative action of which has been noted.

Acknowledgment is made to Major-General H. Marrian Perry, C.B., of the Royal Army Medical College for assistance in providing strains of *B. paratyphosus* A and B, and to my assistants for their continued help.

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CANCER OF THE RECTUM: AN ANALYSIS OF 1000 CASES

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(PLATES LVI-LX)

THIS paper deals with the general characters of rectal cancer, its microscopic structure and manner of spread. It is based on the examination of 1000 tumours removed by radical excision of the rectum.

In each case a record has been kept of the age and sex of the patient; the size, shape, position and microscopic structure of the tumour; the extent of local spread and the presence of lymphatic metastases. In 596 cases a gland dissection was also carried out and each lymphatic gland removed separately for microscopic examination, its position being marked on a scale drawing or photograph. In 539 cases a dissection was also made of the hæmorrhoidal veins, transverse sections being cut to detect any evidence of thrombosis or intravascular growth. No case was included unless the primary tumour appeared to have been completely removed with at least an inch or two of free margin above so that a satisfactory dissection could be made of the tissues surrounding the growth and of its lymphatic field.

The operation specimens were laid open with scissors along the anterior rectal wall and stretched out before fixation. A rectangular frame composed of meccano perforated strips with an adjustable crossbar was used for this purpose (fig. 1). The specimen was secured to the frame by stitching with needle and twine, the sutures being kept taut so as to retain the normal length and breadth. The rectum was then fixed by immersion for one or two days in a tall jar or tank containing 10 per cent. formalin, after which it was removed from the frame and measurements made of the size, shape and position of the growth. For microscopic examination slices were removed through the region of deepest visible extension in the rectal wall or perirectal fat. The glands and vessels were next dissected and the scale drawing prepared. After microscopic examination of the sections the extent of local spread and the position of lymphatic metastases and intravascular growth was marked in the drawing. Finally the gland dissection was photographed, and a photograph of this and the surface view of the tumour were attached to the pathological report. Examples of two cases, one with a single lymphatic metastasis and the other with several, are illustrated in figs. 2 and 3.

General characters of rectal cancer

Sex. Cancer of the rectum is commoner in men than in women. In this series there were 650 males and 350 females. These were

patients who had submitted to surgical treatment and might not constitute a fair sample of the general distribution of rectal cancer, so it is interesting to compare them with the mortality rate for this disease throughout England and Wales. The Registrar-General's statistical review for 1936 reported 3303 deaths in males from cancer of the rectum (excluding the anus) and 2083 in females, a ratio per thousand of 613 : 387. These figures correspond closely with the ratio of 650 : 350 in this series.

Age. The average age at the time of surgical treatment was 57.4 years. Age is of considerable interest in relation to rectal cancer. The disease occurs slightly earlier in women than in men, the mean ages being 55.1 and 58.6 respectively, a significant difference (3.5 ± 0.8). The earlier age of onset is also shown by the fact that 11.2 per cent. of the women were under forty as compared with only 6 per cent. of the men (table I). A similar

TABLE I

Age at time of surgical treatment for rectal cancer

Age group	Both sexes	Males	Females	Percentage of males	Percentage of females
20-39	71	36	35	6.0	11.2
40-59	431	276	155	45.7	49.7
60-80	413	291	122	48.3	39.1
Over 80	2	1	1
Age unknown	83	46	37
Total	1000	650	350
Mean age	57.4 ± 0.4	58.6 ± 0.4	55.1 ± 0.7
Standard deviation	10.2	11.7	10.9

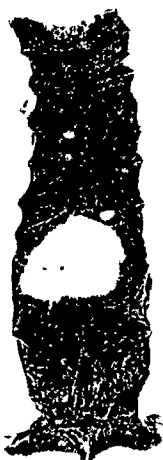
difference is found in the Registrar-General's figures for England and Wales. The mean age of the 2083 women who died of rectal cancer in 1936 was 64.4 years and for the 3303 men 66.0 years.

The age at the time of surgical treatment is also of interest because a more widespread extension of the disease is likely to be found in young patients. For the purpose of measuring extent of spread the presence or absence of lymphatic metastases may be accepted as a fair standard. Analysis showed that, in patients under forty, lymphatic metastases were found in 71.8 ± 4.3 per cent., whereas in the age-group 40-59 they were found only in 50.9 ± 2.4 per cent., a statistically significant difference (table II). This relation between extent of spread and the age of the patient held for both sexes. The observed fact might be attributed to delay in receiving surgical treatment or to a more rapid progress of the disease in young patients. It seems improbable that delay

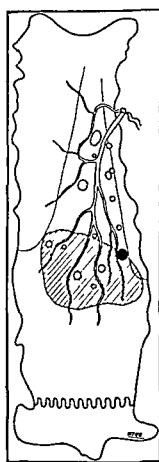
CANCER OF THE RECTUM



FIG 1—Operation specimen stretched on frame for fixation



Surface view

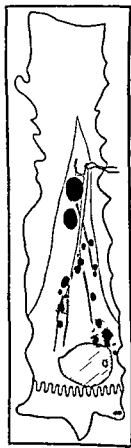


Gland dissection

FIG 2—Method of recording specimens. The position of the tumour is marked in the gland dissection chart by a shaded area. Glands free from metastases are outlined only, gland with metastasis black.



Surface view



Gland dissection

FIG 3—Method of recording specimens. Stippled areas around affected glands signify local extension.

CANCER OF THE RECTUM

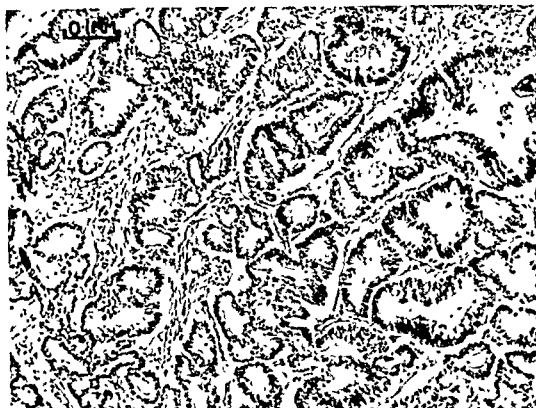


FIG. 4—Carcinoma of rectum. Grade I.

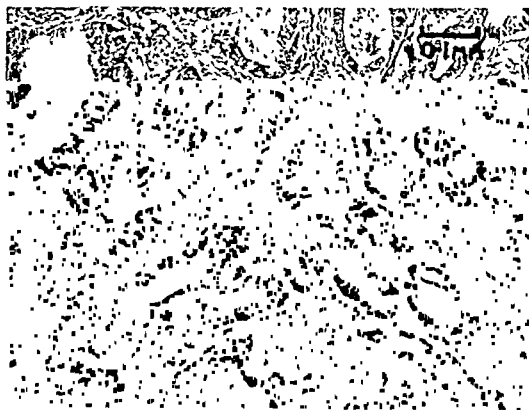


FIG. 5—Carcinoma of rectum. Grade II.

CANCER OF THE RECTUM

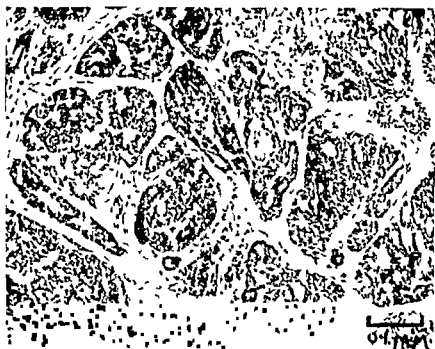


FIG 6—Carcinoma of rectum Grade III

FIG 7—Carcinoma of rectum Grade IV

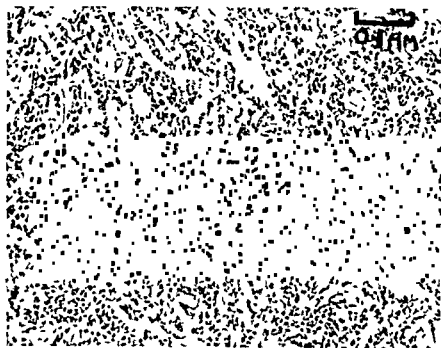


FIG 8—Mucoid carcinoma of rectum



in diagnosis is a sufficient explanation. The most likely explanation is that cancer of the rectum gives rise to metastases more rapidly in young patients than in old.

TABLE II

Relation of lymphatic metastases to age of patient (915 cases)

Age group in years	Cases in each group	Number with metastases	Percentage with metastases
Both sexes			
20-39	71	51	71.8 \pm 4.3
40-59	431	219	50.9 \pm 2.4
60-79	413	190	46.0 \pm 2.4
Females			
20-39	35	26	74.0 \pm 7.4
40-59	155	90	58.0 \pm 3.9
60-79	122	66	54.1 \pm 4.5
Males			
20-39	36	25	69.4 \pm 7.7
40-59	276	129	46.9 \pm 3.0
60-79	291	124	42.6 \pm 2.9

Position. The rectum is naturally divided into two portions by the line of peritoneal reflexion, the lower being the smaller. For purposes of localisation of cancer, however, we arbitrarily recognise lower, middle and upper "thirds", of which the lower third is the shortest and the upper third the longest. Obviously therefore this method underestimates the relative frequency of "lower third" tumours because of the smaller size of this segment of gut. Using these arbitrary divisions it was found that 36.6 per cent. of rectal cancers were in the lower third, 32.6 per cent. in the ampulla (or middle third) and 30.8 per cent. in the upper third.

Size. At the time of surgical treatment most rectal cancers have the shape of oval ulcers slightly longer in transverse than in longitudinal diameter. They usually extend over two or three quadrants of the rectum and measure from two to three inches in diameter from above downwards. Growths less than one inch or more than four inches in diameter are rare. No relationship was found to exist between the size of the tumour and its site of origin.

Multiple malignant tumours

In most cases of rectal cancer treated by radical excision one malignant growth only is found, but associated malignant tumours are fairly common and may be encountered in the following circumstances.

(a) *Multiple primary cancer.* More than one primary focus of carcinoma was found in 29 cases (2.9 per cent.). Four of these were associated with familial polyposis intestini and are classed under that head. In the remaining 25 cases only two carcinomatous tumours were found in each specimen, these being separated by at least an inch or more of normal bowel. Most commonly the two cancers were similar in size and shape and appeared to be of equal duration, but in other cases the second tumour was obviously at an earlier stage of development. A comparison of these 25 cases with the whole series of 1000 cases did not reveal any significant difference as regards sex, age, histology or extent of local, lymphatic or venous spread.

Multiple cancer raises a point of special interest in surgical pathology. The fact that a second malignant growth was found in as many as 2.9 per cent. of cases when only the rectum was examined makes it probable that more cases of multiple intestinal cancer would have been discovered if the whole colon could have been inspected. Therefore some cases of alleged recurrence after excision of the rectum for cancer may be due to a second unsuspected primary growth in another part of the colon and not to a recurrence of the rectal growth.

(b) *Secondary malignant tumours.* Secondary deposits of carcinoma are sometimes found in the mucous membrane of the rectum above or below a primary growth. This is a rare phenomenon, being associated only with massive and continuous growth within veins or widespread lymphatic dissemination. The deposits give rise to rounded ridges or smooth nodular swellings, often umbilicated in the centre (fig. 11), which appear to be due to re-eruption on the surface of growth within small veins in the submucosa (fig. 12). In these cases dissection reveals a continuous cord of malignant tissue extending along the larger hæmorrhoidal veins to their tributaries in the submucosa. This type of secondary malignant tumour is due, in short, to retrograde venous extension.

(c) *Familial polyposis intestini.* The association of cancer of the rectum and colon with familial polyposis is now well established. In this disease the mucosal surface of the rectum and colon is covered by innumerable sessile and pedunculated adenomata. Malignant change generally begins in one or more of these growths and is often multiple.

Seven instances of familial polyposis were met with in this series of 1000 cases of rectal cancer. In one case four separate carcinomas were found in the rectum, in another three, and in two there were two malignant growths. The ages at the time of treatment of these seven cases were 25, 30, 38 (2 cases), 46 (2 cases) and 53, giving a mean age of 39.9 years. This is considerably less than the mean age for the whole series (57.4 years: table I), but as there are only

seven cases in the familial polyposis group the difference cannot be regarded as statistically significant

The features which distinguish rectal cancer associated with polyposis are —(1) a familial history of polyposis and often of intestinal cancer, (2) onset of rectal cancer at an early age; (3) frequency of multiple carcinomata

Histology

On the basis of microscopic structure rectal cancer is divided naturally into two main groups—(1) adenocarcinoma or columnar celled carcinoma and (2) mucoid carcinoma. Some further histological subdivision is desirable because in each group individual tumours vary considerably, some being anaplastic and others relatively well differentiated. Terms such as "highly malignant" or "relatively benign" have long been used by pathologists to express these differences. The system of histological grading introduced by Broders was an attempt to standardise this procedure, and is well adapted for rectal adenocarcinoma provided it is based on a general survey of the tumour and not on a single microscope field. Actually the decision as to the grade of malignancy is dependent more on the general arrangement of the glandular tissue than on the relative number of so called undifferentiated cells. There are of course no sharply defined boundaries to the grades, grading is an artificial division into four arbitrary groups. Some difficulty is likely to be expected therefore in placing tumours which appear to be intermediate in character. In spite of these limitations grading is a useful method of subdividing rectal cancers and yields information of value from the point of view of prognosis, especially in the cases falling within the extreme grades I and IV.

The histological bases of the grading scheme are as follows. Grade I tumours closely resemble an adenoma in which there are signs of active epithelial proliferation. As a rule they can only be recognised as malignant when evidence of invasion or infiltration is found (fig 4). In grade II tumours the cancer cells are more crowded together but still arranged in a fairly regular fashion, one or two layers deep, around glandular spaces. The nuclear material stains deeply and irregular mitotic figures are fairly common (fig 5). The cancer cells of grade III tumours are less differentiated and are arranged in irregularly folded rings often two or three deep around glandular spaces, or in solid clumps. Mitotic figures are more numerous and more atypical than in grade II tumours (fig 6). In grade IV the malignant cells are even more anaplastic and do not form glandular structures but pervade the tissues singly or in small irregular groups and columns (fig 7). Mucoid tumours also vary considerably in degree of differentiation but it is difficult to establish satisfactory standards for grading them and they have all been grouped together (fig 8). The histological classification adopted for this analysis is therefore four grades of adenocarcinoma and one of mucoid. These standards have been applied to the whole series of 1000 cases but fifteen

growths which could not be fitted into this system owing to their atypical histology have been omitted from the present analysis.

Grade II proved to be the largest group. Of the total of 985 cases 66 were in grade I, 559 in grade II, 224 in grade III, 14 in grade IV and 122 were mucoid. The distribution of the grades was approximately the same in each sex, but tumours of high-grade malignancy appeared to be more common in young patients, though the difference did not quite reach the accepted level of significance in the χ^2 test. The average age of patients with grade I tumours was 58.6 ± 1.3 , with grade II 58.2 ± 0.5 , with grade III 56.1 ± 0.7 and with grade IV 47.5 years ± 3.8 . The higher proportion of grade IV tumours in young patients may be associated with the fact that at the time of treatment cancer is often found to have spread more widely in young patients than in the middle-aged or elderly.

The position of the growth in the rectum bore no relation to its histological structure, nor was the size of a tumour any indication of its histological grade. As might be expected there was a close relationship between grade and lymphatic and venous spread. Lymphatic metastases occurred in 24.2 ± 5.3 per cent. of grade I tumours, in 41.3 ± 2.1 per cent. of grade II, in 71 ± 3.0 per cent. of grade III, and in 92.8 ± 6.9 per cent. of grade IV. The incidence in mucoid growths was 65.3 ± 4.3 per cent. (table III). This

TABLE III

Relation of histological grade to lymphatic metastasis (985 cases)

Grade	Number of cases	Number with lymphatic metastases	Percentage with lymphatic metastases
I	66	16	24.2 ± 5.3
II	559	231	41.3 ± 2.1
III	224	159	71.0 ± 3.0
IV	14	13	92.8 ± 6.9
Mucoid	122	80	65.3 ± 4.3
Total . . .	985	499	50.6

orderly increase in the percentage with metastases as the grade advances is obviously unlikely to be due to chance and the χ^2 test confirms this conclusion. Excluding the mucoid cases $\chi^2 = 83$; P less than 0.01.

A similar relationship was found to exist between histological grades and lymphatic permeation or venous spread (tables IV and V). Evidence of lymphatic permeation was found in only 4.4 ± 3.0 per cent. of grade I tumours, but increased to 8.6 ± 5.1 per cent. in grade II, to 30.1 ± 4.2 per cent. in grade III and to 50 ± 15.8 per cent.

in grade IV. Here again the progression of the percentage of cases with lymphatic permeation as the grade advances is unlikely to be due to chance and the χ^2 test confirms this conclusion. Excluding the mucoid cases $\chi^2 = 45$; P less than 0.01.

TABLE IV

Relation of histological grade to lymphatic permeation (539 cases)

Grade	Number of cases	Number with lymphatic permeation	Percentage with lymphatic permeation
I	45	2	4.4 ± 3.0
II	302	26	8.6 ± 5.1
III	113	34	30.1 ± 4.2
IV	10	5	50.0 ± 15.8
Mucoid	69	15	21.7 ± 5.0
Total	539	82	15.2

TABLE V

Relation of histological grade to venous spread (539 cases)

Grade	Number of cases	Number with venous spread	Percentage with venous spread
I	45	2	4.4 ± 3.0
II	302	37	12.2 ± 1.8
III	113	41	36.3 ± 4.5
IV	10	2	20.0 ± 12.7
Mucoid	69	14	20.3 ± 4.8
Total	539	96	17.8

Evidence of venous spread was found in 4.4 ± 3.0 per cent. of grade I tumours, in 12.2 ± 1.8 per cent. of grade II tumours, in 36.3 ± 4.5 per cent. of grade III tumours and in 20.0 ± 12.7 per cent. of grade IV tumours. The incidence of venous spread in cases of mucoid cancer was 20.3 ± 4.8 per cent. Excluding the mucoid cases $\chi^2 = 39$, P less than 0.01.

These considerations emphasise the importance of the histology of a rectal cancer since this may allow an estimate to be made of its rate of growth and may even provide an indication of the extent of local, lymphatic or venous spread.

Spread of rectal cancer

Local spread. In its earliest stage cancer of the rectum takes the form of a small thickening of the mucous membrane or a hard nodule in a pre-existing adenoma or villous papilloma. As the

growth increases in size it gives rise to ulceration. Growths of low grade malignancy (grade I) tend to proliferate on the surface, forming bulky projecting tumours covering a relatively large surface area. Growths of high grade malignancy (grades III and IV) ulcerate early, due to the fact that they penetrate quickly into the rectal muscle and perirectal fat. Ulceration usually commences when the growth has spread through the submucosa into the muscle. It is due primarily to necrosis following vascular obstruction. Deep ulceration may always be taken as a sign that the growth has spread through the muscle into the perirectal tissues.

It is convenient to have some method of measuring local spread and for this purpose all the 1000 cases were divided into three groups—A, B and C—according to the extent of spread. "A"

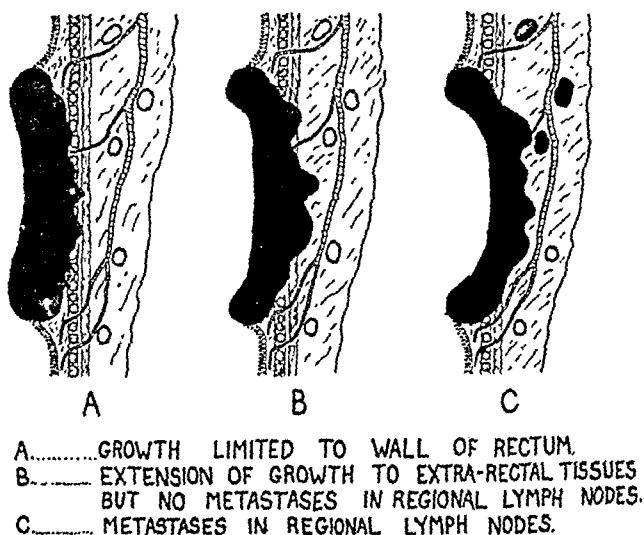


FIG. 9.

cases are those in which the growth was confined to the rectal wall, there being no extension into the extra-rectal tissue and no metastases in the lymphatic glands. In "B" cases the growth had spread by direct continuity into the extra-rectal tissues but the lymphatic glands were free from metastases. Cases were classified as "C" if lymphatic metastases were found (fig. 9).

Of the cases of cancer of the rectum accepted by surgeons as operable, about 15 per cent. were found to be A cases, 35 per cent. B and 50 per cent. C. This proportion remained constant from year to year but was influenced slightly by social grade. Of the 1000 patients in this series 148 were private patients sufficiently wealthy to pay the fees of nursing homes; the remainder were of the hospital class. The proportion of A cases was 19.6 per cent. amongst the private patients as compared with 14 per cent. for

the hospital class. The difference (5.6 per cent with a standard error of 3.16) is not statistically significant but suggests that possibly cancer of the rectum may be diagnosed at an earlier stage in the rich than in the poor.

The extent of local spread was not related in any special way to the position of the growth. The proportion of A, B and C cases was approximately the same for tumours in the upper third, ampulla and lower third of the rectum, and no significant relationship was found to exist between size of tumour and extent of spread.

As might be expected, however, a close connection was found between histological grade and extent of local spread. Grading may be regarded as an approximate method of measuring the pace of growth of a tumour and the A, B and C classification as a measurement of the distance reached. The two methods of measurement are naturally interrelated, as is shown by the analysis in table VI. The A cases (limited to rectal wall) contained the

TABLE VI

Relation of extent of spread to histological grade (985 cases)

	Grade I		Grade II		Grade III		Grade IV		Mucoid	
	No	Percent	No	Percent	No	Percent	No	Percent	No	Percent
144 A cases	28	19.4	97	67.4	8	5.5	0	0	11	7.7
342 B	20	5.8	233	68.1	57	16.7	1	0.3	31	9.1
499 C	16	3.3	232	46.4	159	31.8	13	2.7	79	15.8

largest percentage of grade I growths (slow growing tumours) whereas the C cases (with metastases) contained the highest percentage of the more malignant grades. The appropriate statistical test shows that the differences of grading revealed between the categories A, B and C are more than could be attributed to chance. Including mucoid cases $\chi^2 = 128$, P less than 0.01.

Venous spread. The extension of rectal cancer by venous channels has received relatively little attention in routine pathological investigations, yet in view of the frequency of secondary growths in the liver, venous spread is obviously of the first importance. Of course data as to venous spread obtained in this way have not the same precision as information concerning local spread or lymphatic metastases. The extent of local spread and the position of lymphatic metastases can be determined from examination of the operation specimen but cancerous growth within veins may not remain fixed. If dissection reveals cancerous deposits within veins, then this is evidence of probable venous

dissemination, but the non-discovery of intravascular growth does not exclude the possibility of venous spread, since emboli may already have been carried away in the blood stream leaving no trace behind.

Evidence of extension of rectal cancer within the lumen of hæmorrhoidal veins was found in 17·8 per cent. of the 539 cases in which the veins were dissected and examined microscopically. This usually took the form of a solid cord detectable by palpation, and extending only a short distance. Less commonly massive thrombosis, with solid fixed intravascular growth, was found to have extended several inches along the hæmorrhoidal veins (fig. 10), a condition usually associated with secondary malignant tumours in the rectal mucosa above and below the primary growth (fig. 11). Dissection showed these to consist of carcinomatous deposits within the venules of the submucosa, a continuous cord of malignant growth joining these small tributaries to the large hæmorrhoidal veins lying in the perirectal fat (fig. 12).

The mean age of patients in whom venous spread was found was $56\cdot6\pm1\cdot2$ years as compared with $58\cdot2\pm0\cdot6$ years for those without evidence of venous spread, a difference which is without statistical significance. No instance of venous spread was found in A cases, from which it may be inferred that as a general rule venous dissemination does not commence until the tumour has spread by direct continuity into the perirectal fat. Venous spread was most commonly found in large tumours situated in the upper third of the rectum.

As might be expected venous spread was closely related to lymphatic spread. Of the cases with glandular metastases, $21\cdot6\pm2\cdot4$ per cent. showed venous spread as compared with $13\cdot7\pm2\cdot1$ per cent. of cases without glandular metastases, a difference which is statistically significant. As a rule the larger the number of lymphatic metastases the more frequently was venous spread found. The close proximity of the large hæmorrhoidal veins to the chain of lymphatic glands made it seem likely that in some cases the vascular invasion might have been an extension from an adjacent glandular metastasis. This explanation could only apply to cases associated with lymphatic metastases, but in 36 out of the 96 cases exhibiting venous spread no lymphatic metastases were found, and in these the invasion of the veins must have been direct from the primary growth.

Lymphatic spread. The lymphatic glands were examined for metastases in all the 1000 cases. In the first 400 a note was made stating whether there were few or many metastases, but the last 600 operation specimens were dissected, each gland numbered and the size and position of the metastases marked on a scale drawing. It was soon noticed that apart altogether from carcinomatous

CANCER OF THE RECTUM



FIG. 12.—Extension of a rectal carcinoma along a small vein in the submucosa

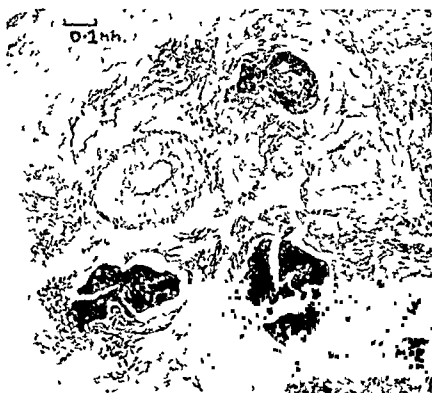


FIG. 13.—Extension of rectal carcinoma by permeation of lymphatic channels accompanying hemorrhoidal vessels

metastases the number of lymphatic glands found by dissection of operation specimens was very variable, ranging from nil to more than fifty, with an average of seventeen. The chief factor influencing the number of glands was found to be the size of the primary growth. Glands were generally few in number with small growths and most numerous with large bulky tumours.

It may be stated as a general rule that lymphatic metastases are rarely found at the stage before a rectal cancer has spread by direct continuity into the perirectal fat. Exceptions to this rule are found in growths of high grade malignancy. Out of 400 cases in which special attention was paid to this point, 14 instances only were found of glandular metastases whilst the primary growth appeared limited to the rectum, an incidence of 3.5 per cent.

Lymphatic metastases were found in 505 of the 1000 cases. There was a significant difference in the incidence of glandular metastases in the two sexes, lymphatic spread being more common in women. Metastases were found in 57.7 per cent. of the females and 46.6 per cent. of the males (difference 11.1, S.E. 3.2). In both sexes the percentage of cases with glandular metastases was higher in young patients than in old (table II). There was no significant relationship between the size or position of the growth and the presence of lymphatic metastases, but as already mentioned there was a close relationship between lymphatic metastases and histological grade, metastases being most frequent in growths of high grade malignancy (table III).

The actual number of lymphatic metastases is a point of some interest. In the majority of cases in which glandular metastases were found only a few glands were affected, the average for the series being 4.7. One metastasis only was found in 21.9 per cent., two or less metastases in 37 per cent. and three or less in 51.5 per cent. One may assume from this that as a rule the spread of rectal cancer from gland to gland is a slow process. If it were rapid, one would expect either no metastases or many. A fairly close relationship was found to exist between the number of glandular metastases and the histological grade of the primary tumour. Cases with several lymphatic metastases were almost invariably grades III or IV or mucoid growths.

Evidence of lymphatic permeation (a continuous cord of growth within the lymphatic channels extending from gland to gland) was found in 15 per cent. of 539 specially investigated cases (fig. 13). The mean age of patients with lymphatic permeation was five years younger than those with no signs of lymphatic permeation— 53.2 ± 1.2 as compared with 58.2 ± 0.5 years, a statistically significant difference.

There was, as might be expected, a very close relation between lymphatic permeation and the presence of lymph-gland metastases.

Evidence of lymphatic permeation was very rarely found in either the intra- or extramural lymphatic channels unless several metastases were also present in the lymphatic glands.

Lymphatic permeation in rectal cancer should not be regarded as an alternative to the normal method of spread by cancerous emboli but as an exaggerated expression of the normal method consequent on a high grade of malignancy. In these cases malignant proliferation is so rapid that the cancer cells grow along at the same time as they pass along the lymphatic channels, forming a continuous cord extending from gland to gland.

Summary

The main conclusions to be drawn from this analysis of 1000 cases of cancer of the rectum treated by rectal excision are as follows.

1. Rectal cancer is commoner in men than in women. The average age of onset for women is earlier than for men.

2. At the time of surgical treatment the disease is likely to have spread more extensively in young patients than in the middle-aged or elderly.

3. All regions of the rectum may be affected approximately to the same extent, though probably the incidence decreases slightly on passing from the anus towards the pelvic colon.

4. When surgically removed, most cancers have the form of oval ulcers 2-3 inches in diameter and extending over two or three quadrants of the rectum.

5. Where more than one malignant tumour is found this may be due either to multiple primary carcinoma or to secondary tumours resulting from venous extension. Multiple primary carcinoma is especially common in cases of polyposis intestini.

6. Histological grading is a useful method of subdividing adenocarcinoma of the rectum because it may give an indication of the extent of local spread. Tumours of high grade malignancy are most frequent in young patients. A close relationship exists also between the histology of rectal cancer and the extent of lymphatic and venous spread.

7. In about 15 per cent. of cases of rectal cancer regarded as operable the growth is found to be still restricted to the rectal wall (A cases). In about 35 per cent. the growth has spread by direct continuity into the perirectal fat but has not yet caused lymphatic metastases (B cases). Lymphatic metastases are found approximately in 50 per cent. of all cases treated by radical excision (C cases).

8. Evidence of extension of rectal cancer within the lumen of the hæmorrhoidal veins can be found in approximately 18 per cent. of all operation specimens.

9. Lymphatic metastases are commoner in women than in men and are found more frequently in the young than in the elderly. Metastases rarely occur before the primary growth has spread by direct continuity into the perirectal fat. In the majority of cases exhibiting lymphatic spread only a few glands are affected. Evidence of lymphatic permeation is found in about 15 per cent. of all cases, these being mostly growths of high grade malignancy.

This work has been carried out in the pathology department of St Mark's Hospital with the aid of a grant from the British Empire Cancer Campaign. I am under special obligation to my senior laboratory assistant, H. J. R. Bussey, B.Sc., for his skilled technical help. All the surgeons at St Mark's Hospital have taken an interest in this work and I am indebted to each for advice, especially to Mr J. P. Lockhart Mummery and Mr W. B. Gabriel. For statistical help I am also indebted to Dr A. Bradford Hill. I hope that this work may be regarded as a continuation and amplification of Mr W. Ernest Miles' pioneer work on the pathology of cancer of the rectum.

SHORT ARTICLES

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THE EFFECT OF IRON DEFICIENCY ON THE FRAGILITY OF RAT ERYTHROCYTES

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It is now twenty years since Vallery Radot and Lhéritier (1919) showed that the fragility of the mammalian erythrocyte to hypotonic saline varied in different species. In man the normal and pathological variations have been fairly well established (Daland and Worthley, 1934-35, Randall 1937-38), but little work on animals has been attempted. The object of this paper is to record the effect of experimental iron deficiency on the fragility of rat erythrocytes, which normally are slightly more fragile than those of man.

Experimental procedure

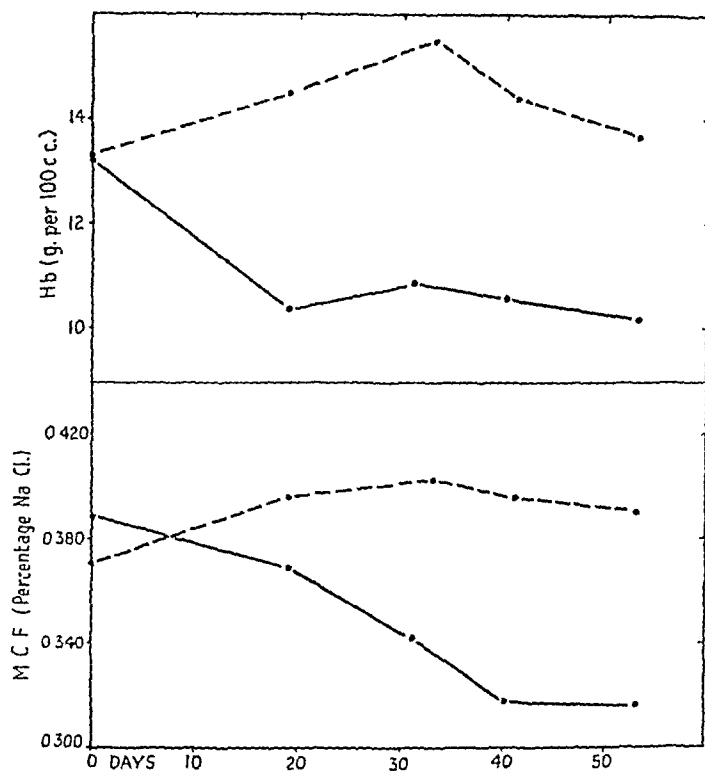
Ten white rats, six weeks old and of approximately equal weight (45 g.), were chosen from two litters and divided into an experimental and a control series. They were fed on a diet containing little iron—white bread, milk, wheat germ and cod liver oil, but the control series received a supplement of iron and copper—1 mg. of iron as ferrous ammonium sulphate and 0.025 mg. of copper as copper sulphate per rat daily. They were kept in adjacent cages for eight weeks and haemoglobin and fragility estimated at approximately ten day intervals. At the end of the experiment the animals were killed, the erythrocytes per cmm. were counted and haemoglobin, fragility and packed cell volume estimated.

Blood was obtained by section of the tails after warming. 0.1 to 0.15 c.c. was sufficient. This was received into small tubes containing 0.01 mg. of heparin and was immediately gently mixed with the aid of a fine sealed glass capillary.

Haemoglobin was estimated by the Haldane carbon monoxide method, 100 per cent corresponding to 13.8 g. Hb per 100 c.c. Fragility was estimated by a slight modification of a technique previously described (Dacie and Vaughan, 1938), and is suitable for small amounts of blood. The blood was aerated until bright red by gentle stirring and 0.0125 c.c. added by a small automatic pipette to 0.3 c.c. of a restricted range of hypotonic saline kept at 0°C in crushed ice. Haemolysis was measured quantitatively after centrifuging by comparison with standards of known haemoglobin content made from other blood. The range of saline solutions was selected so that the saline concentration causing 50 per cent lysis (median corpuscular fragility, or M.C.F.) could be read off a quantitative graph of haemolysis. Packed cell volume was estimated using 100 mm. capillary haematocrit tubes, which were centrifuged at 3000 r.p.m. for 45 minutes.

Results

The means of the observations in the two groups of five animals are recorded in the accompanying figure and table. In the rats receiving no supplement of iron the hæmoglobin fell from a mean initial value of 13.2 g.



Effect of iron deficiency on rat erythrocytes.

Continuous lines=iron-deficient rats. Interrupted lines=control series.

TABLE

Effect of iron deficiency on rat erythrocytes

	Initial		Final	
	Iron-deficient rats	Controls	Iron-deficient rats	Controls
Hæmoglobin (g. per 100 c.c.)	13.2* (11.1-15.2)†	13.3 (11.9-14.4)	10.2 (9.1-12.3)	13.7 (13.1-14.1)
Mean corpuscular hæmoglobin (M.C.H.) (γγ)	10.3	15.1
Packed cell volume (per cent.)	38.0	43.2
Fragility (M.C.F.) (NaCl per cent.)	0.388* (0.330-0.429)†	0.371 (0.332-0.405)	0.317 (0.288-0.363)	0.391 (0.382-0.407)
t P	0.828 0.4-0.5		5.72 <0.01	

* Mean value of series.

† Range of series.

to 10.2 g per 100 c.c. of blood, and the mean M.C.F. fell from 0.388 to 0.317 per cent NaCl. The mean haemoglobin of the rats which received an iron and copper supplement rose from 13.3 to 13.7 g per 100 c.c. of blood, and the M.C.F. rose from 0.371 to 0.391 per cent NaCl. The final mean corpuscular haemoglobin (M.C.H.) was 10.3 $\gamma\gamma$ in the iron deficient rats compared with 15.1 $\gamma\gamma$ in the controls. As the probability (P, Fisher, 1938) of such a difference as is shown in the corpuscular fragilities of the two groups of rats arising by chance is less than 1 in 100, there can be no doubt of its statistical significance. Initially the 10 rats could be considered as a sample of the same population, but at the end of the experiment it was highly probable that there was a real difference between the two groups.

Comment

This simple experiment shows that young rats fed on a diet deficient in iron produce erythrocytes with a low haemoglobin content and that these cells are unusually resistant to haemolysis with hypotonic saline. This is similar to the behaviour of human cells under similar conditions (Daland and Worthley, 1934-35, Barrett, 1938), and it is clear that the haemoglobin content of a cell, by affecting cell volume, has a controlling influence on its fragility.

It is generally agreed that the determining factor responsible for variation in fragility is cell shape (Haden, 1934, Vaughan, 1937, Castle and Daland, 1937), a cell being more fragile the closer it approximates to a spheroidal as opposed to a discoidal form, i.e. the larger its volume relative to its surface area, and species variation is usually explained on this basis (Castle and Daland). The experiment now recorded shows that fragility in the rat may vary, and experimentally rat erythrocytes may become less fragile than those of man (0.317 compared with 0.366 per cent * NaCl).

Summary

1 Iron deficiency lowers the haemoglobin content and decreases the fragility of the erythrocytes of the young rat.

2 The fragility of rat erythrocytes is thus a variable characteristic of the species.

My best thanks are due to the Will Edmonds Clinical Research Fund for financial assistance.

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* This is the mean human M.C.F. calculated from Dacie and Vaughan's (1938) series of fifty adults.

ROUTINE BLOOD COUNTS WITH HEPARINISED BLOOD

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For a complete cytological examination of the blood it is necessary to take venous blood and add an anticoagulant. With the Wintrobe technique (Wintrobe, 1933) it is possible to estimate the erythrocyte sedimentation rate, hæmatocrit percentage and icterus index in the same tube. The anticoagulant now recommended by Wintrobe and Landsberg (1935) is a mixture of ammonium and potassium oxalate, which is isotonic with the blood and produces no alterations in any of the corpuscular constituents. Heparin has certain advantages over the oxalate mixture. There is a much greater margin of permissible error in the relation between volume of specimen and amount of anticoagulant, and one can also estimate corpuscular fragility and the majority of chemical constituents, including urea and the cations, tests which are not possible with blood containing the two oxalates. Although heparin appears to be the physiological anticoagulant (Jorpes, 1939), it has been reported to produce flocculation of white cells and platelets and irregular readings of the hæmatocrit and the erythrocyte sedimentation rate. Many of the investigations have been done on normal blood. We have therefore compared the counts on blood taken from the same venipuncture into oxalate and heparin specimen tubes in 34 clinical cases, most of which were abnormal, including a number of leukæmias. A concentration of 1 mg. of heparin is sufficient for 5-50 c.c. of blood, and in our counts 0.2 c.c. of a 1 per cent. solution was put into a specimen tube and dried out in a desiccator. Whereas the blood specimens taken into oxalate tubes were always accurately measured, a good deal of latitude was allowed with the specimens taken into heparin tubes, the quantities varying from 10 to 20 c.c. Counts were usually made within two hours and were never postponed overnight. The averages obtained from these 34 counts are almost identical, except for a slightly higher value for the sedimentation of heparinised blood. It would therefore appear that heparinised blood is quite suitable for routine cytological examinations.

Anticoagulant	R.B.C.	Hb	W.B.C.	Hæmatocrit	E.S.R.
Oxalate . . .	3.545 m.	74 per cent.	40,842	31.6	27.0
Heparin . . .	3.543 „	75 „	40,733	31.5	29.3

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THE SEROLOGICAL RELATIONSHIP BETWEEN THE VIRUSES
OF JAPANESE AND ST LOUIS ENCEPHALITIS

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For some time before his death the late J R Perdrau had been engaged on a serological study of the viruses of Japanese and St Louis encephalitis. This note is compiled from the data found in his note books in the hope that the information it contains may be of use to others working on the same or allied subjects

P P L

The two viruses employed throughout the investigation were supplied by Dr L T Webster of the Rockefeller Foundation, and they were maintained by passage in mice. Two consignments of virus direct from Japan proved to be quite inactive on test soon after receipt. Every effort was made to keep the two viruses separate. Different isolation rooms in the animal house were allotted for animals inoculated with each virus and in the laboratory it was the rule that the two viruses were never worked with on the same day.

Sera were prepared by hyperimmunising *rhesus* monkeys and rabbits. From the notes it is clear that the rabbit hyperimmune sera had a very similar action on the whole, but were much more potent than the monkey sera. The technique which gave the most potent serum was as follows. Rabbits were given four (increasing) doses of virus subcutaneously at weekly intervals. The initial dose was usually half a mouse brain emulsified in saline and the fourth dose two mouse brains. After a rest of three weeks a final large dose of virus (two mouse brains) was given and one week later the animals were bled out and the serum harvested. The sera were not inactivated but were stored in the cold until required.

The sera were tested and compared by mixing constant amounts of serum with diminishing dilutions of virus, falling in multiples of ten. The serum virus mixtures were kept at room temperature for an hour or else incubated at 37°C for 2, 5, 9 or 20 hours. Each serum virus mixture was then inoculated intracerebrally into six mice, which were observed for 14 days. Those which survived this period were regarded as having survived indefinitely and were destroyed. The standard virus was the supernatant fluid from a lightly centrifuged 1 per cent emulsion of mouse brain in digest broth.

Each neutralising experiment required adequate controls and it is clear that the effect of incubation on the virus was important and the possibility was open that anti mouse antibodies present in the hyperimmune sera, in addition to the antiviral antibodies, might produce an effect. It was found that virus incubated at 37°C with saline deteriorated rapidly and was almost inactive after 20 hours. The addition of normal rabbit serum stabilised the virus and there was only a small loss of activity after 20 hours' incubation at 37°C. Serum from a rabbit (779) hyperimmunised with normal mouse brain did not appear to affect the virus adversely. After 5 hours' incubation, serum virus mixtures of this type were still active with virus diluted to 10^{-5} , and in another experiment after 20 hours at 10^{-4} dilution.

In view of these findings there was included in each neutralising experiment a series of virus dilutions mixed with normal rabbit serum. These were incubated alongside the virus-antiserum mixtures and formed controls of virus potency. Each serum was tested for the number of fatal doses of virus it could neutralise. There was no standard serum of reference and as the virus varied somewhat from one experiment to another only broad conclusions can be drawn.

On reviewing the experiments as a whole it would seem that more virus was neutralised on incubation than at room temperature and the longer the incubation the greater the neutralisation. In one experiment where the same mixtures were tested after 2 hours' and 20 hours' incubation at 37° C. the result was decisively in favour of the longer incubation. When different times of incubation were used with different batches of virus, the results were not so easy to interpret, but on the whole the longer period gave better neutralisation. It seems clear that incubation at 37° C. for 2 hours or more was the method chosen for the comparison of sera.

Sera from rabbits hyperimmunised with Japanese virus not only neutralised this virus, but also had a striking effect on St Louis virus. Conversely the anti-St Louis sera had a neutralising effect on the homologous virus and also upon the heterologous Japanese virus. This is well shown by the results given in tables I and II.

TABLE I

Comparison of the neutralising power of antisera prepared against Japanese virus and St Louis virus when tested against Japanese virus

Japanese virus dilution	Antiserum 777 (anti-J.)	Antiserum 787 (anti-St L.)	Normal rabbit serum
10 ⁻²	6, S, S, S, S, S	9, S, S, S, S, S	...
10 ⁻³	S, S, S, S, S, S	S, S, S, S, S	4, 4, 4, 4, 6, 6
10 ⁻⁴	S, S, S, S, S, S	S, S, S, S, S, S	4, 4, 4, 4, 6, 6
10 ⁻⁵	4, 6, 6, 6, 6, S
10 ⁻⁶	S, S, S, S, S, S

All serum-virus mixtures incubated at 37° C. for 20 hours.

In columns 2-4, figures indicate day of death of mouse ; S = survival.

It seems clear that serum prepared against the virus of either Japanese encephalitis or St Louis encephalitis will neutralise about 1000 fatal doses of the virus of Japanese encephalitis. When these two sera were tested against the virus of St Louis encephalitis a very similar result was obtained ; both sera neutralised about 100 fatal doses of virus (table II).

The experiments quoted above have been selected because they show some of the largest effects recorded in the notes and these two sera seem to have been the most potent obtained. The results are none the less typical in that other sera and even these same sera under different conditions seem to show approximately the same power to neutralise the homologous or the heterologous viruses. It is true that some experiments with the rabbit antisera appear to show a slightly greater power to neutralise the homologous virus and yet others that the heterologous virus is more easily neutralised. Trying to view the experiments as a whole it seems fair to conclude that the two types of antisera neutralise the homologous and heterologous viruses with equal facility to about the same end-point. It is inevitable that considerable variation should occur in biological tests of

this kind and it is very difficult to get the true end-point with unstable biological products and limited numbers of animals.

TABLE II

Comparison of the potency of antisera prepared against the viruses of Japanese and St Louis encephalitis when tested with the virus of St Louis encephalitis

St Louis virus dilution	Antiserum 777 (anti-J.)	Antiserum 787 (anti St L.)	Normal rabbit serum
10 ⁻²	10, S, S, S, S, S	S, S, S, S, S, S	
10 ⁻³	S, S, S, S, S, S	S, S, S, S, S, S	
10 ⁻⁴	S, S, S, S, S, S	S, S, S, S, S, S	5, 5, 5, 7, 7, S
10 ⁻⁵	...		S, S, S, S, S, S
10 ⁻⁶			S, S, S, S, S, S

In columns 2-4, figures indicate day of death of mouse, S = survival

The sera prepared in monkeys gave on the whole very similar answers, except that the sera were much weaker and only occasionally neutralised 100 fatal doses of virus. With the monkey sera there were also more instances of the heterologous virus being neutralised to a greater degree than the homologous; but this may have been associated with the lesser potency of these sera and a vaguer end-point.

These results differ from those recorded by Webster (1938) and by the Japanese workers (Kawamura *et al.*, 1936; Kudo *et al.*, 1937), who find the two viruses to be distinct, as judged by serological tests with convalescent sera. Using hyperimmune serum, prepared in monkeys, Webster also found the two viruses to be different, with perhaps a trace of overlap. The reason for this divergence in the experimental results remains obscure. The matter is made still more difficult by the fact that Webster found that mice immunised to Japanese encephalitis were susceptible to St Louis virus and conversely. Perdrau's notes show that 3 mice, proved to be immune to Japanese virus intracerebrally, resisted St Louis virus completely; but subsequently succumbed to louping-ill when tested with that virus.

Conclusions

1. Antisera of considerable potency can be prepared in rabbits by hyperimmunisation with the viruses of Japanese and St Louis encephalitis.
2. Such antisera neutralise the homologous and heterologous viruses to approximately the same extent.
3. It appears that the viruses of Japanese and of St Louis encephalitis are antigenically similar, when examined by means of hyperimmune sera.

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EFFECT OF A DIET SUPPLEMENTED BY FRESH LIVER ON
INDUCED SKIN TUMOURS IN INBRED MICE

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A strain of mice has been described and termed IF (Bonser, 1938) in which benign skin tumours induced either by tar or by pure carcinogenic substances appeared earlier than in other mice tested; but there was a longer interval between wart appearance and the development of malignancy in these mice than in those of other strains. As these characteristics appeared to be constitutional it was thought that an examination of the effect of the addition of fresh liver to the diet might yield interesting results.

Maisin and François (1928), using hybrid mice, observed an acceleration of benign and malignant tar tumours when they supplemented a basal diet two or three times per week with fresh or desiccated liver. Watson (1933) observed an increased carcinogenic response in tar-treated hybrid mice when the diet was supplemented with fresh ox or horse liver. This increased response was shown (1) by the earlier appearance of benign warts, and (2) by the development of larger numbers of warts and epitheliomata. But the time interval between the first tar treatment and the development of malignancy was not reduced by the addition of liver. Kreyberg (1938), using inbred mice of the white label strain, confirmed Watson's findings but noted that the earlier benign tumour formation in the liver-fed mice was due to the appearance of warts which subsequently regressed.

Details of technique

1. *Diet.* The scheme described by Watson was used, *i.e.* one group of 37 mice received a basal diet consisting of four parts breadcrumbs and one part rolled oats mixed to a paste with water, while the diet of the other group of 50 mice was supplemented by the addition of 18 per cent. ox liver obtained fresh from the abattoir twice weekly. The daily diet for each animal was approximately 8 g. In addition, all the mice received marmite and cod liver oil once weekly and lettuce twice weekly.

2. *Mice.* These belonged to the 15th, 16th and 17th generations of inbreeding of the IF strain and approximately one-third of each group were corresponding litter mates. An epidemic of ectromelia caused a high mortality in the early weeks of the experiment, so that at the time of the appearance of the first wart (8 weeks) the groups consisted of 34 liver-fed mice (17 of each sex) and 26 control mice (11 females and 15 males).

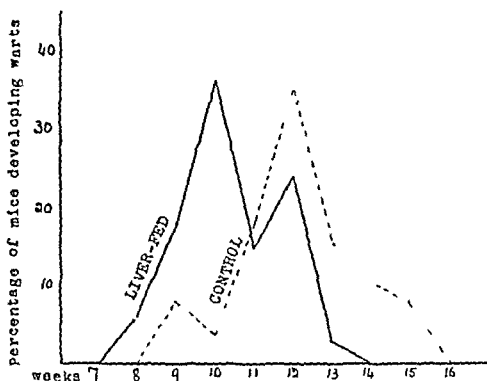
3. *Carcinogen.* A solution of 0.3 per cent. 3:4-benzpyrene* in benzene was applied once weekly to the skin of the back between the shoulder blades with a camel-hair brush until a malignant tumour was detected. This had been found previously to be a weaker carcinogenic agent than tar or a similar solution of methylcholanthrene.

Results of the experiment

1. *Benign tumours.* In the liver-fed group, all the warts appeared between the 8th and 13th weeks, in the control group between the 9th and 15th weeks. There were no deaths during this period and none of the warts regressed.

* Supplied by Messrs Light, Wraybury, Bucks.

If the percentage of new tumours appearing each week is examined (see graph), it is seen that the only difference is a lag of two weeks in the development of warts in the control group. In other words, 97 per cent of liver fed mice had developed warts by the end of the 12th week, in contrast with only 65 per cent of the controls.



Development of benign tumours in liver fed and control groups of mice

2. *Malignant tumours* As the estimation of malignancy by means of palpation had previously been found to be satisfactory (Bonser), this method was used here. Warts in 6 liver fed mice failed to become malignant, in 2 cases due to extensive ulceration of the skin, in 4 cases due to early death. Only one wart in the control group failed to become malignant. When the interval between wart appearance and development of malignancy was considered, great individual variation was found, ranging from 2 to 17 weeks in the liver fed mice and from 2 to 13 weeks in the controls. The average interval was 10.5 ± 0.8 and 8.4 ± 0.6 weeks respectively. This difference just attains the conventional level of statistical significance.

Discussion

The addition of fresh liver to the diet of II¹ mice, which are highly susceptible to carcinogenic agents applied to the skin, has resulted in a slight acceleration of the appearance of benign tumours, compensated by a corresponding delay in the development of malignant tumours. These effects are very slight and are of a lesser order than those obtained by other workers, who used hybrid mice or inbred mice with a late fat response. The effects are, however, similar in kind to those described by other workers and thus it seems that the development of infiltration is a process less susceptible to extrinsic influence than the process of initiation of tumour growth. It would also seem that genetic constitution is capable of influence by extrinsic factors, as has indeed been shown in other fields. The survival rate in both groups of mice after the early mortality from ectromelia was so good that it is unlikely that the acceleration of benign tumours could have been due to improved general health of the liver group.

In this experiment the comparison of the results in the two groups of mice was aided by the fact that all were derived from the same inbred generations, a considerable proportion being derived from the same litters. It is, however, of interest to compare the present rate of tumour induction with that in a previous experiment (Bonser) where inbred mice of generations 12 and 13 were used. In the latter the range of appearance of benign tumours was 9-28 weeks, the majority occurring between the 13th and 20th weeks. The average interval between wart appearance and the development of malignancy was 12.2 weeks. In the present experiment the range was 9-15 weeks and the average interval 8.4 weeks.

Three factors may have been concerned in determining these differences: (1) the diet of the control mice in the present experiment was less adequate than in the previous experiment; (2) the temperature of the room was not controlled in the present experiment and while varying considerably was probably consistently lower by 10° F. than in the previous experiment, where it was thermostatically controlled at about 65° F.; (3) the mice used in the present experiment were further inbred. It is impossible to say which of these factors is responsible for the great differences in time of development of skin tumours in the two experiments. It is, however, satisfactory to note that the mice of the IF strain now used are at least as susceptible to carcinogens applied to the skin as those used in the earlier experiments and there may even be a tendency to increased susceptibility with further inbreeding. Benign tumours appear consistently very much earlier in these mice than in any others at present in use in this laboratory.

Summary

1. By supplementing the diet of IF mice, which are highly susceptible to carcinogenic agents applied to the skin, with fresh ox liver, a slight acceleration of benign tumour formation was obtained. There was, however, a corresponding delay in the development of malignant tumours. These differences are of a lesser order but of the same kind as those described by other workers, who used hybrid mice or inbred mice with a late tar response.

2. An acceleration of both benign and malignant tumour formation was noticed in the mice used in this experiment when compared with mice of the same strain in a previous experiment. Several factors may have been concerned in determining these differences.

3. IF mice continue to be much more susceptible to carcinogens applied to the skin than any other mice in use in this laboratory.

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BOOKS RECEIVED

Viruses and virus diseases

By T M RIVERS 1939 California, Stanford University Press
London, Humphrey Milford, Ltd (Oxford University Press) Pp 133,
34 text figs 14s 6d

In the first of his Lane Medical Lectures for 1939 Dr Rivers describes in detail the investigation of lymphocytic choriomeningitis which led to the discovery of the virus which causes this disease. It serves as an example of the way in which such diseases are investigated and their nature determined and so as a fitting introduction to the remaining four lectures. Lecture II is devoted to the pathology of virus diseases and gives a very clear account of the author's views on the nature of inclusion bodies, the inflammatory and proliferative changes seen in various virus infections and the relation of viruses to tumour growth, the last a most judicious and critical contribution. This lecture contains some beautiful illustrations whose value is rather diminished by printing on unsuitable paper. In lecture III Dr Rivers discusses problems relating to immunity, in lecture IV the nature of viruses and in lecture V prophylactic and therapeutic treatment. It is most valuable to have in this short compass a statement of the views of one of the foremost workers in this field on the present position of these various problems, particularly as they are so cogently argued and so judiciously appraised. All the most recent techniques of study, even the electron microscope, are considered, but it is comforting to the biologist to find that the big molecule has not yet supplanted the living organism. The predominating feature of Dr Rivers's review is that he considers that the time for broad generalisation has not yet arrived on any of these problems and that in certain cases, as for example the nature of viruses, it may never come. "I still hold," he says on page 99, "the position taken in 1932 that some of the viruses may be minute, highly parasitic microorganisms, the midgets of the microbial world, capable of reproduction only within susceptible host cells, that others may represent forms of life more or less unfamiliar to us, and that still others may be fabrications of their host cells aided by the processes of autocatalysis. What life is and where the transition from the non-living to the living takes place, if it does, in the scheme just set forth is not known. Furthermore, the transition may be so gradual that it will be difficult for investigators to assign the particular point at which it occurs."

With such a conclusion one might have expected to see some reference to Boycott's views but there is none, though those of other workers are freely referred to. It is a most valuable book both for the beginner, for the pathologist of wide interests and for the expert.

Pathogenic microorganisms

By W H PARK and ANNA W WILLIAMS 11th edition, 1939 London
Baillière, Tindall and Cox Pp 1056, 247 text figs and 13 plates (10 in colour) 40s

This volume appears almost exactly forty years after the publication of the first edition. Previous editions have come along so regularly and acceptably that ordinarily it would be sufficient to draw the attention of the large number who are interested to the arrival of a new one. This particular edition is, however, something of a milestone. It appears from the preface that Dr Williams, for the first time, did not share in its

production, and its preparation was the final task of Dr Park before his death in April 1939. Appropriately there are included a portrait of Park and a brief biographical sketch.

The volume contains 1056 pages of text as compared with the 867 of its predecessor. The subject matter has been rearranged under 8 headings, three of which are new and by collaborators, filterable viruses by Shaeffer, pathogenic yeasts, moulds and actinomycetes by Weidmann and pathogenic protozoa by Bercovitz. Other new features are chapters on bacterial metabolism by Blanchard and bacterial variability by Hadley. These and other rearrangements bring the number of chapters up to 61 as compared with 55 in the 10th edition. There are a few photographs of the great, and the enormous identification table, which always tended to slip out of the older editions and was often taken out and used as a laboratory decoration, has now been included in the ordinary pagination. One notes a certain disinclination to discuss the modern serology of the intestinal organisms and the pasteurella group, there is a rather inadequate discussion of the types of *C. diphtheriæ*, and the account of Rickettsial diseases is somewhat meagre.

The value of this book, however, lies largely in the personal experience which it records—experience not confined to bacteria, as may be judged from the remark on page 455 that "The physician must have intelligence to use advantageously laboratory findings."

Elementary microtechnique

By H. ALAN PEACOCK. 2nd edition, 1940. London: Edward Arnold & Co. Pp. viii and 330. 21 text figs. 9s.

This book by a former head of the department of biology of Cheltenham Grammar School is an excellent little manual to put into the hands of a laboratory assistant or advanced student. It gives a clear and up-to-date account of the simpler histological procedures, as well as detailed reasons for the various steps and many useful hints about different materials and the little difficulties that arise in handling them. There is a chapter (no. I) on the cell and another (no. III) on the microscope. Chapter II is a summary of principles and technique, of which the section on dehydration is particularly good. Chapters IV and V describe in detail a series of methods of increasing complexity for making temporary and permanent preparations of botanical and zoological tissues, and should be most useful. Mallory's connective tissue stain however is rather a difficult one for routine work; perhaps Masson's acid fuchsin (or Biebrich scarlet)-light green method might be better; this can be used after the iron hæmatoxylin of Anderson and of Weigert, which are given, or that of Janssen, which is more stable. Chapters VI (on methods for specific materials) and VII (on formulæ) are arranged alphabetically, making the rather rudimentary index sufficient. Details about the more advanced methods are sometimes sketchy, for example those for elastic tissue, and there are occasional contradictions. (On p. 139 it is stated that Mallory stains elastic tissue red, on p. 144 blue and on p. 262 not at all.) Stock Zenker (p. 290) should be made up minus acetic, and the formulæ for Carnoy (p. 234), Flemming (p. 247), Heidenhain's iron hæmatoxylin (p. 253) and Lugol's iodine (p. 261) should be revised. Finally there are two chapters on the source and culture of materials and their preservation, and there must be few who will not find some fresh tips here. Any criticisms in fact are of details and the book will be welcomed with gratitude by many teachers and heads of departments.

INDEX OF SUBJECTS

	PAGE		PAGE
ANTIFERNS, bacterial spores as	235	<i>Clostridium noly</i> rapid method of distinguishing from other gas gangrene bacteria	167
" " " "	481	<i>Clostridium uelchii</i> blood changes and post mortem findings following intravenous inoculation of sheep with culture filtrates of <i>Cl uelchii</i> , types A, C and D	251
" " " "	287	survey of types in soil and in intestine	271
" " " "	69	COAGULASE production as a criterion for the classification of the staphylococci	53
" " " "	od of gas	COMPLEMENT FIXATION with Shope rabbit papilloma virus	169
gangrene bacteria	167	CONJUNCTIVITIS infection of	221
BLOOD COUNTS with heparinised blood	541	" " " "	243
BLOOD CULTURE <i>in vitro</i> of leucocytes from human bone marrow	145	atmosphere	491
BLOOD SUGAR of rabbits, effect of <i>H pertussis</i> and <i>Br bronchiseptica</i> on	11	DESENSITISATION of tuberculous guinea pigs, effect of	89
BLOOD SUGAR, post mortem changes in	473	DIPHTHERIA infection of white mice with	243
BLOOD VOLUME of mouse	372	ENCEPHALITIS, serological relationship between viruses of Japanese and St Louis	545
BOVE MARROW, culture <i>in vitro</i> of leucocytes from human	145	ENDOCYTOPTIC due to a new species of	497
BOOKS RECEIVED	185, 385, 551	ENTOMOLOGY	per
BRONCHITIS, <i>Haemophilus influenzae</i> and influenza virus in relation to histology of catarrhal influenzal bronchitis in mice infected with influenza virus	317	mental type	the
<i>Brucella bronchiseptica</i> effect of extracts on the blood sugar of rabbits	11	immature rabbit	507
CANCER of rectum	527	EPILEPSIA congenital diverticulum of left ventricle in a case of	61
CARBON DIOXIDE METABOLISM and staphylococcus infection in rabbits	1	<i>Erysipelothrix rhusiopathiae</i> experimental infection in swine	323
CARCINOGENESIS, EXPERIMENTAL effect on induced skin tumours of a diet supplemented by fresh liver	548	ERYTHROCYTES, effect of iron deficiency on fragility of rat	541
first effects on mouse skin of some polycyclic hydrocarbons	463	FIBROMA occurrence of virus III in infectious fibroma of rabbits	227
histology of rat's liver during carcinogenesis by butter yellow	393	FIBROSIS of liver in heart failure	221
CARRIER STATE chemotherapy of the experimental typhoid carrier state in the immature rabbit	507	FOL KURLOFF CELL, sex hormones and the	201
CEREBELLAR HYPOPLASIA and degeneration in Hereford calves	455	FRAGILITY OF RED CELLS effect of iron deficiency on	541
CHEMOTHERAPY a phenanthridinium compound in the treatment of experimental infections with <i>T</i>	371	GAS GANGRENE BACTERIA rapid method	167
" " "	" "	GEY	" "
<i>Leishmania tropica</i>	171	mice	17
of experimental staphylococcal infection in mice with drugs of sulphanilamide group	421	renal lesions in pure lines of mice	25
of the experimental typhoid carrier state in the immature rabbit	507	GLYCOLYSIS, post mortem	473
CHOLERA vibrios characteristic haptens and antigen of rugose races of	160	GOITRE, histology of toxic nodular	198
		GUINEA PIG effect of desensitisation of tuberculous guinea pigs	89

	PAGE		PAGE
<i>Haemophilus aphrophilus</i> N. Sp., endocarditis due to	497	MOUSE: passive protection of mice with immune horse serum against living staphylococci	439
<i>Haemophilus influenzae</i> in relation to bronchitis	317	pleuropneumonia-like organisms in mice capable of producing pneumonia	409
<i>Haemophilus pertussis</i> : effect of extracts on blood sugar of rabbits	11	renal lesions in pure lines of mice	25
HEART: congenital diverticulum of left ventricle in epiloia	61		
HORMONES: pathological changes in sex organs after prolonged administration of sex hormones to female rats	295	OBITUARY NOTICES	
sex hormones and the Foà-Kurloff cell	201	William Howel Evans (portrait)	177
HYPOPLASIA of cerebellum in Hereford calves	455	John Gray	182
		PAPILLOMA VIRUS: complement fixation with Shope rabbit	169
IMMUNITY: passive protection of mice with immune horse serum against staphylococci	439	PHLEBOSCLEROSIS	121
to influenza in mice	37	PIG: polyarthritides in swine	323
INCLUSIONS: occurrence of intranuclear inclusions in cultures of foetal leptomeninges	53	PLEUROPNEUMONIA-LIKE ORGANISMS: occurrence in normal mice	409
INFLUENZA in mice, immunity and antibody to	37	POLYARTHRITIS in swine	323
INFLUENZA VIRUS in relation to bronchitis	317	PROCEEDINGS OF THE SOCIETY	197
INFLUENZAL BRONCHITIS, histology of, in mice infected with influenza virus	31		
IRON DEFICIENCY: effect on red cell fragility	541	RABBIT: carbohydrate metabolism and staphylococcus infection in rabbits	1
KIDNEY: renal lesions in pure lines of mice	25	chemotherapy of the experimental typhoid carrier state in the immature rabbit	507
		complement fixation with Shope papilloma virus	169
<i>Leishmania tropica</i> , comparative efficiency of certain drugs and dyes in killing cultures of	171	effect of extracts of <i>H. pertussis</i> and <i>Br. bronchiseptica</i> on blood sugar	11
LEUCOCYTES: culture <i>in vitro</i> of leucocytes from human bone marrow	145	occurrence of virus III in infectious fibroma and transplantable sarcoma of rabbits	227
LIVER: fibrosis in heart failure	221	vaccinia virus in corneal cells of	279
histology of, during carcinogenesis by butter-yellow	393	RAT: effect of iron deficiency on fragility of erythrocytes	541
incidence of tumours of, in a pure line of mice	17	histology of rat's liver during carcinogenesis by butter-yellow	393
		histopathology of rats following lack of different constituents of the vitamin B ₂ complex	199
MENINGES: occurrence of intranuclear inclusions in cultures of foetal leptomeninges	53	pathological changes in sex organs after prolonged administration of sex hormones to female rats	295
MOUSE: a phenanthridinium compound in the treatment of experimental infections with <i>T. congolense</i>	371	RECTUM, cancer of	527
blood volume of	372		
chemotherapy of experimental staphylococcal infection with drugs of sulphanilamide group	431	SARCOMA: occurrence of virus III in transplantable sarcoma of rabbits	227
effect on induced skin tumours in inbred mice of a diet supplemented by fresh liver	548	SEROLOGY: effect of blood serum on growth of tubercle bacilli in the depth of liquid media	111
first effects on mouse skin of some polycyclic hydrocarbons	463	SEX HORMONES and the Foà-Kurloff cell	201
histology of influenzal bronchitis in mice infected with influenza virus	31	pathological changes in sex organs of female rats after prolonged administration of	295
immunity and antibody to influenza in mice	37	SHEEP: blood changes and post-mortem findings following intravenous inoculation with culture filtrates of <i>Cl. welchii</i> , types A, C and D	251
incidence of tumours of liver and other organs in a pure line of mice	17	SHOPE RABBIT: PAPILLOMA VIRUS, complement fixation with	169
infection of white mice with the three types of <i>C. diphtheriae</i>	243	SKIN: first effects on mouse skin of some polycyclic hydrocarbons	463
		SPORES as antigens	235
		STAINING METHODS: a controllable modification of Mallory's trichromic staining method	381

- | | PAGE | | PAGE |
|--|------|---|------|
| HOLMAN, H. H., W. S. GORDON, JAMES STEWART and A. WILSON TAYLOR, blood changes and post-mortem findings following intravenous inoculation of sheep with culture filtrates of <i>Cl. welchii</i> , types A, C and D | 251 | McLEOD, J. W., and J. GORDON, a simple and rapid method of distinguishing <i>Cl. novyi</i> (<i>B. oedematiens</i>) from other bacteria associated with gas gangrene | 167 |
| HOLMAN, W. L., S. H. JACKSON and T. F. NICHOLSON, carbohydrate metabolism and staphylococcus infection in rabbits | 1 | MAIER, IRENE, G. S. WILSON and HERTA SCHWABACHER, effect of the desensitisation of tuberculous guinea-pigs | 89 |
| HOWIE, J. W., and J. CRUICKSHANK, bacterial spores as antigens | 235 | MARTIN, L. C., histology of toxic nodular goitre | 198 |
| HOYLE, L., complement fixation with the Shope rabbit papilloma virus | 169 | MULDER, J., <i>Haemophilus influenzae</i> and influenza virus in relation to bronchitis | 317 |
| INNES, J. R. M., DOROTHY S. RUSSELL and A. J. WILSDON, familial cerebellar hypoplasia and degeneration in Hereford calves | 455 | NICHOLSON, T. F., S. H. JACKSON and W. L. HOLMAN, carbohydrate metabolism and staphylococcus infection in rabbits | 1 |
| INNES, J. R. M., and J. W. WHITTICK, thrombo-angiitis obliterans in a horse | 377 | NORMAN, R. M., and A. L. TAYLOR, congenital diverticulum of the left ventricle of the heart in a case of epiloia | 61 |
| ISRAELS, M. C. G., the culture <i>in vitro</i> of leucocytes from human bone marrow | 145 | OAKLEY, C. L., and G. HARRIET WARRACK, immunity and antibody to influenza in mice | 37 |
| JACKSON, S. H., T. F. NICHOLSON and W. L. HOLMAN, carbohydrate metabolism and staphylococcus infection in rabbits | 1 | OAKLEY, C. L., and G. HARRIET WARRACK, the blood volume of the mouse | 372 |
| JOHNSON, ELSIE W. M., and J. L. HAMILTON-PATERSON, post-mortem glycolysis | 473 | ODDY, J. G., and D. G. EVANS, the effects produced by toxic and non-toxic extracts of <i>H. pertussis</i> and <i>Br. bronchiseptica</i> on the blood sugar of rabbits | 11 |
| JOHNSTONE, K. I., and J. GORDON, bactericidal action of normal sera | 483 | ORR, JOHN W., the histology of the rat's liver during the course of carcinogenesis by butter-yellow (<i>p</i> -dimethylaminoazobenzene) | 393 |
| KEOGH, E. V., and R. T. SIMMONS, cultural methods as an aid in type differentiation of group A haemolytic streptococci | 137 | PAGEL, WALTER, the effect of blood serum on the growth of tubercle bacilli in the depth of liquid media | 111 |
| KHAIRAT, OMAR, effect of a CO ₂ atmosphere on blood cultures | 491 | PEDEN, D., CLARA RAVEN and H. D. WRIGHT, bacteriological examination of water supplies | 287 |
| KHAIRAT, OMAR, endocarditis due to a new species of <i>Haemophilus</i> | 497 | PERDRAU, J. R., serological relationship between the viruses of Japanese and St Louis encephalitis | 545 |
| KITCHING, J. S., and L. N. FARRELL, passive protection of mice with immune horse serum against living staphylococci | 439 | PIASECKA-ZEYLAND, E., and J. ZEYLAND, influence of the size of the infecting dose on the course of tuberculosis | 153 |
| KORENCHESKY, V., and K. HALL, pathological changes in the sex organs after prolonged administration of sex hormones to female rats | 295 | PIASECKA-ZEYLAND, E., and J. ZEYLAND, influence of the virulence of infecting bacilli on the course of tuberculosis | 156 |
| LEDINGHAM, J. C. G., sex hormones and the Foà-Kurloff cell | 201 | PULLINGER, B. D., first effects on mouse skin of some polycyclic hydrocarbons | 463 |
| LENDRUM, ALAN C., and DAVID MCFARLANE, a controllable modification of Mallory's trichromic staining method | 381 | RAVEN, CLARA, D. PEDEN and H. D. WRIGHT, bacteriological examination of water supplies | 287 |
| LI, PEI-LIN, adaptation in veins to increased intravenous pressure, with special reference to the portal system and inferior vena cava | 121 | | |
| MCFARLANE, DAVID, and ALAN C. LENDRUM, a controllable modification of Mallory's trichromic staining method | 381 | | |

	PAGE		PAGE
ROBB, J V M, C H BROWNING and PAUL BROWNING, a phenanthridinium compound of Morgan and Walls as a chemotherapeutic agent in experimental infections with <i>T. congolense</i>	371	TAYLOR, A WILSON, W S GORDON, JAMES STEWART and H H HOLMAN, blood changes and post mortem findings following intravenous inoculation of sheep with culture filtrates of <i>Cl. welchii</i> , types A, C and D	251
RUSSELL DOROTHY S, and CATHERINE F FISCHMANN, occurrence of intra nuclear inclusions in cultures of foetal leptomeninges	53	WAINMAN, L M, and G M BONSER, effect on induced skin tumours in inbred mice of a diet supplemented by fresh liver	548
RUSSELL DOROTHY S, J R M INNES and A J WILSON, familial cerebellar hypoplasia and degeneration in Hereford calves	455	WARRACK, G HARRIET, and C L OAKLEY, immunity and antibody to influenza in mice	37
SCHWABACHER, HERTA, G S WILSON and IRVINE MAIER, effect of the desensitisation of tuberculous guinea pigs	89	WARRACK, G HARRIET, and C L OAKLEY, the blood volume of the mouse	372
SENTEKJI, H A, a comparison of the efficiency of certain drugs and dyes in killing cultures of <i>Leishmania tropica</i>	171	WATTS, P S, studies on <i>Erysipelothrix rhusiopathia</i>	355
SHORT, R H D, the vasa vasorum of the femoral vein	419	WHITL, P BRUCE, the characteristic haptene and antigen of rugose races of cholera and El Tor vibrios	160
SRIMONS, R T, and E V KEOGH cultural methods as an aid in type differentiation of group A haemolytic streptococci	137	WHITE P BRUCE, a heat labile somatic protein antigen (H L S P) of vibrios	165
STEWART, JAMES W S GORDON, H H HOLMAN and A WILSON TAYLOR blood changes and post mortem findings following intravenous inoculation of sheep with culture filtrates of <i>Cl. welchii</i> , types A, C and D	251	WHITTICK J W and J R M INNES, thrombo angitis obliterans in a horse	377
STRAUB M, histology of catarrhal influenzal bronchitis and collapse of the lung in mice infected with influenza virus	31	WILSON A J, J R M INNES and DOROTHY S RUSSELL, familial cerebellar hypoplasia and degeneration in Hereford calves	455
		WILSON, G S, HERTA SCHWABACHER and IRVINE MAIER effect of the desensitisation of tuberculous guinea pigs	89
		WITTS L J routine blood counts with heparinised blood	544
		WRIGHT H D, CLARA RAYN and D PEDEN bacteriological examination of water supplies	287
		ZEYLAND, J, and E PIASECKA ZEYLAND, influence of the size of the infecting dose on the course of tuberculosis	153
TAYLOR, A L, and R M NORMAN, congenital diverticulum of the left ventricle of the heart in a case of epiloia	61	ZEYLAND, J, and E PIASECKA ZEYLAND, influence of the virulence of infecting bacilli on the course of tuberculosis	156
TAYLOR A WILSON, and W S GORDON, survey of the types of <i>Cl. welchii</i> present in soil and in the intestinal contents of animals and man	271	ZINNEMANN, K, infection of white mice with the three types of <i>C. diphtheriae</i>	243